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| (54) Title: ANALOGUES OF hGH-RH (1-29)NH ₂ HAVING ANTAGONISTIC ACTIVITY (57) Abstract Synthetic analogues of hGH-RH(1-29)NH ₂ having substitutions of various amino acids and acylated at the N-terminus, and exhibiting prolonged antagonistic duration. Embodiments include analogues of the formula: X-R ¹ -R ² -R ³ -R ⁴ -R ⁵ -R ⁶ -Thr-R ⁸ -Ser-Tyr-R ¹¹ -R ¹² -Val-Leu-R ¹⁵ -Gln-Leu-Ser-R ¹⁹ -R ²⁰ -R ²¹ -Leu-Leu-Gln-Asp-Ile-R ²⁷ -R ²⁸ -R ²⁹ , wherein X is nil, H, Ac, IAc, BrProp, For, Ibu, Nac, 2-Nac, 1- or 2-Npt, 1- or 2-Npr or Aqc; R ¹ is Tyr, His, Glu or Glt; R ² is D-Arg, D-Cit, D-Har, D-Lys or D-Orn; R ³ is Asp, Ala or Gly; R ⁴ is Ala or Gly; R ⁵ is Ile, Ala or Gly; R ⁶ is Phe, Ala, Pro, Tpi, Nal or Phe(Y), in which Y is F, Cl, Br, NO ₂ , CH ₃ or OCH ₃ ; R ⁸ is Asn, Ser, Val, Ile, Ala, Abu, Nle or Aib; R ¹¹ is Arg, D-Arg or Cit; R ¹² is Lys, D-Lys Cit or Ala; R ¹⁵ is Gly, Ala, Abu or Gln; R ¹⁹ is Ala or Abu; R ²⁰ is Arg, D-Arg or Cit; R ²¹ is Lys, D-Lys or Cit; R ²⁷ is Met, Nle or Abu; R ²⁸ is Ser, Asn, Asp or Abu; R ²⁹ is Agm, Arg-NH ₂ , Arg-OH, Cit-NH ₂ , Cit-OH, Har-NH ₂ or Har-OH; provided that when R ¹ is Glt, X is nil and when X is H, R ¹⁵ is other than Gly, and pharmaceutically acceptable acid addition salts thereof. | | |

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ANALOGUES OF hGH-RH(1-29)NH₂ HAVING ANTAGONISTIC ACTIVITY
FIELD OF THE INVENTION

This invention was made in part with Government support from the Medical Research Service of the Veterans Affairs Department. The
5 Government has certain rights in this application.

The present invention relates to novel synthetic peptides which inhibit the release of growth hormone from the pituitary in mammals, and to therapeutic compositions containing these novel peptides.

10

BACKGROUND OF THE INVENTION

Growth Hormone ("GH") is a peptide having 191 amino acids which stimulates the production of numerous different growth factors IGF-I and so promotes growth of numerous tissues (skeleton, connective tissue, muscle
15 and viscera) and physiological activities (raising nucleic acid and protein synthesis and lipolysis, but lowering urea secretion).

Release of GH is under the control of releasing and inhibiting factors secreted by the hypothalamus. The primary releasing factor is growth
20 hormone releasing hormone ("GH-RH"); human growth hormone-releasing hormone ("hGH-RH") is a peptide having 44 amino acids. The novel peptides of the present invention relate to analogues of hGH-RH having only residues 1 through 29 ("hGH-RH(1-29)NH₂"), i.e., to analogues of the peptide which has the amino acid sequence:

25 Tyr-Ala-Asp-Ala-Ile⁵-Phe-Thr-Asn-Ser-Tyr¹⁰-Arg-Lys-Val-Leu-Gly¹⁵-
Gln-Leu-Ser-Ala-Arg²⁰-Lys-Leu-Leu-Gln-Asp²⁵-Ile-Met-Ser-Arg²⁹-NH₂

GH has been implicated in several diseases. One disease in which GH is involved is acromegaly, in which excessive levels of GH are present. The
30 abnormally enlarged facial and extremity bones of this disease can be treated by administering a GH-RH antagonist.

Further diseases involving GH are diabetic retinopathy and diabetic nephropathy. The damage to the retina and kidneys respectively in these diseases, believed to be due to GH, results in blindness or reduction in kidney function. This damage however can be prevented or slowed by
5 administration of an effective GH-RH antagonist.

In an effort to intervene in these disease and other conditions, some investigators have attempted to control GH levels by using somatostatin, one inhibitor of GH release. However, somatostatin, if administered alone,
10 does not suppress GH or IGF-I levels to a desired degree. If administered in combination with a GH-RH antagonist, somatostatin would improve suppression of IGF-I levels much better.

Other workers have investigated various modifications of GH-RH to
15 elucidate the relationship of the structure of GH-RH to its activity in an effort to provide synthetic congeners with improved agonistic or antagonistic properties. (Synthesis may be by solid phase method, described in US Patent 4,914,189, or in liquid phase, as described in US Patent 4,707,541.) Thus, in one study, it was found that synthesizing GH-
20 RH without its N-terminus residue -- i.e., forming hGH-RH(2-44) -- results in an analogue having GH releasing activity which is only 0.1% that of GH-RH. By contrast, synthesizing a GH-RH analogue without its residues 30 through 44 -- i.e., synthesizing hGH-RH(1-29)NH₂ -- results in an analogue which retains 50% or more of the potency of native hGH-RH. Synthesizing
25 even shorter analogues -- e.g., GH-RH(1-28)NH₂ or GH-RH(1-27)NH₂ -- resulted in substantially lower bioactivity. These results indicate that residues 1 and 29 are important to the bioactivity of GH-RH.

In another study, it was found that acetylating the N-terminus amino
30 acid residue of GH-RH or replacing it with a D-isomer -- thus forming [Ac-Tyr¹]GH-RH or [D-Tyr¹]GH-RH-- lowers the ability of the analogues to release

GH to 2-3% that of GH-RH. These analogues also have less affinity in vitro for GH-RH binding sites. By contrast, acetylation of the alpha amino group of residue 1 in hGH-RH(1-29)NH₂ -- thus forming [AcTyr¹]hGH-RH(1-29)NH₂ -- is found to raise the in vivo potency over that of GH-RH by ten fold or 5 more.

In further studies, it was found that [Ac-Tyr¹,D-Arg²]hGH-RH(1-29)NH₂ ant-agonizes the activation of rat anterior pituitary adenylate cyclase by hGH-RH(1-29)NH₂. The same peptide was found to block the action of GH-
10 RH on its receptors in the pituitary and hypothalamus, and to inhibit the pulsatile growth hormone secretion.

Several reported modifications to GH-RH have resulted in agonistic activity. US Patent 4,659,693 discloses agonists of hGH-RH(1-29) having
15 the formula: R¹-R²-Asp-Ala-Ile-Phe-Thr-Asn-Ser-Tyr-Arg-Lys-Val-Leu-Gly-Gln-Leu-Ser-Ala-Arg-Lys-Leu-Leu-Gln-Asp-Ile-R²⁷-Ser-Arg-NH₂, wherein R¹ is H, Tyr or His; R² may be various residues; and R²⁷ is Nle. These agonists are said to stimulate release of growth hormone releasing factor ("GRF") and so to be suitable in pharmaceutical compositions. ("GRF" is merely a
20 synonym for GH-RH, and the latter abbreviation is used hereinafter, despite use of GRF in US 4,659,693 and other publications.)

US Patent 4,914,189 discloses other analogues of GH-RH which are agonists. In these agonists, the N-terminus group Q¹CO-, where Q¹ signifies
25 certain omega or alpha-omega substituted alkyl groups, may be Tyr or des-amino-Tyr; the C-terminus group NH-Q², where Q² signifies certain lower omega-guanidino-alkyl groups, may be Arg; and R²⁷ may be Nle. These analogues are said to be extremely potent stimulants of GH release and to enjoy high resistance to in vivo enzymatic degradation due to the omega-
30 guanidino-lower alkyl group at the C-terminus.

Published application WO 91/16923 reviews earlier attempts to alter the secondary structure of hGH-RH by modifying its amino acid sequence. These earlier attempts include: replacing Tyr¹, Ala², Asp³ or Asn⁸ with their D-isomers; replacing Ser⁹ with Ala to enhance amphiphilicity of the region; and
5 replacing Asn⁸ with L- or D-Ser, D-Arg, Asn, Thr, Gln or D-Lys. Certain of these modifications are said to enhance GH releasing activity. WO 91/16923 also states that replacing Asn⁸ with Ala induces an enormous increase in GH releasing activity. The peptides said to have this benefit have the formula: [R¹, R², Ala⁸, R¹⁵, Nle²⁷]hGH-RH(1-29)-NH₂, where R¹ is Dat
10 or A-R¹, where A is lower acyl or benzyl and R¹ includes Tyr and His; R² is Ala, D-Ala or N-Me-D-Ala (N-Methyl-D-Ala); and R¹⁵ may include Gly, Ala or Aib. One preferred embodiment has R^{8,9,15} as Ala. It is noted that R⁸ in this publication is never Asn. Pharmaceutical compositions for enhancing growth are further disclosed.

15

European Patent Application Serial No. O 413 839 A, filed August 22, 1989, assigned to the same assignee as the present application, discloses analogues of hGH-RH(1-29)-NH₂ said to have enhanced release of GH. The analogues of this application replace residues 1, 2, 8, 12, 15, 27, 28 and
20 29 as follows: R¹ may be Tyr or Dat; R² may be L or D Ala; R⁸ may be Asn or Ser; R¹² may be L or D isomers of Lys, Arg or Orn; R¹⁵ may be Gly or Ala; R²⁷ may be Nle; R²⁸ may be Asp, Asn or Ser; and R²⁹ may be Agm. However, residue 6 is never replaced: it is always Phe.

25 Yet another modification of hGH-RH was disclosed in US Patent 5,183,660, where GH-RH was conjugated with polyethylene glycol derivatives. The resulting conjugate was said to exhibit decreased antigenicity, delay in biological clearance in vivo and physiological activity over a longer time.

30

In several of these investigations, it was found that variants of the hGH-RH agonistic analogues had antagonistic, rather than agonistic, activity. Thus, in US 4,659,693 (where R² may be certain D-Arg residues substituted with alkyl groups), when R¹ is H, the hGH-RH analogues are said to act as antagonists. Similarly, in WO 91/16923, discussed above, if R² in the analogues is D-Arg, and R⁸, R⁹, and R¹⁵ are substituted as indicated above, antagonistic activity is said to result. These antagonistic peptides are said to be suitable for administration as pharmaceutical compositions to treat conditions associated with excessive levels of GH, e.g., acromegaly.

10

The antagonistic activity of the hGH-RH analogue "[Ser⁹-Ψ[CH₂-NH]-Tyr¹⁰]hGH-RH(1-29)" of US Patent 5,084,555 was said to result from the pseudopeptide bond (i.e., a peptide bond reduced to a [CH₂-NH] linkage) between the R⁹ and R¹⁰ residues. (It is noted that although this patent employed the seemingly redundant "Ψ[CH₂-NH]" formula for the pseudopeptide bond, actually only one such linkage had been introduced into the peptide.) However, the antagonistic properties of [Ser⁹-Ψ[CH₂-NH]-Tyr¹⁰]hGH-RH(1-29) were said to be inferior to a conventional antagonist, [N-Ac-Tyr¹, D-Arg²]GH-RH(1-29)-NH₂.

20

SUMMARY OF THE INVENTION

There is provided a novel series of synthetic analogues of hGH-RH(1-29)NH₂. These analogues inhibit the activity of endogenous hGH-RH, and therefore prevent the release of growth hormone. This inhibition is believed to result from replacement of various amino acids and acylation with aromatic or nonpolar acids at the N-terminus of GH-RH(1-29)NH₂. The analogues exhibit prolonged antagonistic duration.

Specifically, the invention relates to peptides comprising the formula:

X-R¹-R²-R³-R⁴-R⁵-R⁶-Thr-R⁸-Ser-Tyr-R¹¹-R¹²-Val-Leu-R¹⁵-
Gln-Leu-Ser-R¹⁹-R²⁰-R²¹-Leu-Leu-Gln-Asp-Ile-R²⁷-R²⁸-R²⁹

30

wherein

- X is nil, H, Ac, IAc, BrProp, For, Ibu, Nac, 2-Nac, 1- or 2-Npt, 1- or 2-Npr or Aqc,
R¹ is Tyr, His, Glt or Glu,
5 R² is D-Arg, D-Cit, D-Har, D-Lys or D-Orn,
R³ is Asp, Ala or Gly,
R⁴ is Ala or Gly,
R⁵ is Ile, Ala or Gly,
R⁶ is Phe, Ala, Pro, Tpi, Nal, or Phe(Y), in which Y is F, Cl, Br, NO₂, CH₃ or
10 OCH₃,
R⁸ is Asn, Ser, Val, Ile, Ala, Abu, Nle, or Aib,
R¹¹ is Arg, D-Arg or Cit,
R¹² is Lys, D-Lys, Cit or Ala,
R¹⁵ is Gly, Ala, Abu or Gln,
15 R¹⁹ is Ala or Abu,
R²⁰ is Arg, D-Arg or Cit,
R²¹ is Lys, D-Lys or Cit,
R²⁷ is Met, Nle or Abu,
R²⁸ is Ser, Asn, Asp or Abu,
20 R²⁹ is Agm, Arg-NH₂, Arg-OH, Cit-NH₂, Cit-OH, Har-NH₂ or Har-OH,
provided that when R¹ is Glt, X is nil, and when X is H, R¹⁵ is other than
Gly,
and pharmaceutically acceptable acid addition salts thereof.

- 25 Among the preferred embodiments are peptides wherein X is H and
R¹⁵ is Abu; or wherein X is Nac, For, or Ibu, R¹ is Tyr or His, R² is D-Arg or
D-Cit, R³ is Asp, R⁴ is Ala, R⁵ is Ile, R⁶ is Phe(pCl) or Nal, R¹¹ is Arg, R¹² is
Lys, R¹⁵ is Abu or Ala, R¹⁹ is Ala or Abu, R²⁰ is Arg, R²¹ is Lys, R²⁷ is Nle,
R²⁸ is Ser or Asp, and R²⁹ is Agm or Arg-NH₂. Three very preferred
30 embodiments have the formulae:

Nac⁰-Tyr-D-Arg²-Asp-Ala-Ile-Phe(pCl)⁶-Thr-Asn-Ser-Tyr-Arg-Lys-Val-Leu-
 Abu¹⁵-Gln-Leu-Ser-Ala-Arg-Lys-Leu-Leu-Gln-Asp-Ile-Nle²⁷-Ser-Agm
 ("Peptide 18")

Nac⁰-Tyr-D-Arg²-Asp-Ala-Ile-Nal⁶-Thr-Asn-Ser-Tyr-Arg-Lys-Val-Leu-Abu¹⁵-
 5 Gln-Leu-Ser-Ala-Arg-Lys-Leu-Leu-Gln-Asp-Ile-Nle²⁷-Ser-Agm
 ("Peptide 32")

Nac⁰-Tyr-D-Cit²-Asp-Ala-Ile-Phe(pCl)⁶-Thr-Asn-Ser-Tyr-Arg-Lys-Val-Leu-
 Abu¹⁵-Gln-Leu-Ser-Ala-Arg-Lys-Leu-Leu-Gln-Asp-Ile-Nle²⁷-Ser-Agm
 ("Peptide 34").

10 Under well-established convention, these may be abbreviated as follows:

[Nac⁰,D-Arg²,Phe(pCl)⁶,Abu¹⁵,Nle²⁷]hGH-RH(1-28)Agm Peptide 18

[Nac⁰,D-Arg²,Nal⁶,Abu¹⁵,Nle²⁷]hGH-RH(1-28)Agm Peptide 32

[Nac⁰,D-Cit²,Phe(pCl)⁶,Abu¹⁵,Nle²⁷]hGH-RH(1-28)Agm Peptide 34

15 Four especially preferred embodiments have the formulae:

Nac⁰-Tyr-D-Arg²-Asp-Ala-Ile-Phe(pCl)⁶-Thr-Asn-Ser-Tyr-Arg-Lys-Val-Leu-
 Abu¹⁵-Gln-Leu-Ser-Ala-Arg-Lys-Leu-Leu-Gln-Asp-Ile-Nle²⁷-Ser-Arg-NH₂
 ("Peptide 1")

Nac⁰-His¹-D-Arg²-Asp-Ala-Ile-Phe(pCl)⁶-Thr-Asn-Ser-Tyr-Arg-Lys-Val-Leu-
 20 Abu¹⁵-Gln-Leu-Ser-Ala-Arg-Lys-Leu-Leu-Gln-Asp-Ile-Nle²⁷-Ser-Arg-NH₂
 ("Peptide 5")

Ibu⁰-Tyr-D-Arg²-Asp-Ala-Ile-Phe(pCl)⁶-Thr-Asn-Ser-Tyr-Arg-Lys-Val-Leu-
 Abu¹⁵-Gln-Leu-Ser-Ala-Arg-Lys-Leu-Leu-Gln-Asp-Ile-Nle²⁷-Ser-Agm ("Peptide
 19").

25 For⁰-Tyr-D-Arg²-Asp-Ala-Ile-Phe(pCl)⁶-Thr-Asn-Ser-Tyr-Arg-Lys-Val-Leu-
 Abu¹⁵-Gln-Leu-Ser-Ala-Arg-Lys-Leu-Leu-Gln-Asp-Ile-Nle²⁷-Ser-Agm ("Peptide
 38").

These may be represented by well-accepted convention respectively as follows:

30 [Nac⁰,D-Arg²,Phe(pCl)⁶,Abu¹⁵,Nle²⁷]hGH-RH(1-29)NH₂ Peptide 1

[Nac⁰,His¹-D-Arg²,Phe(pCl)⁶,Abu¹⁵,Nle²⁷]hGH-RH(1-29)NH₂ Peptide 5

| | |
|---|------------|
| [Ibu ⁰ ,D-Arg ² ,Phe(pCl) ⁶ ,Abu ¹⁵ ,Nle ²⁷]hGH-RH(1-28)Agm | Peptide 19 |
| [For ⁰ ,D-Arg ² ,Phe(pCl) ⁶ ,Abu ¹⁵ ,Nle ²⁷]hGH-RH(1-28)Agm | Peptide 38 |

It is noted that the amino acid residues from 30 through 44 of the native GH-RH molecule do not appear to be essential to activity; nor does their identity appear to be critical. Therefore, it appears that the addition of some or all of these further amino acid residues to the C-terminus of the hGH-RH(1-29)-NH₂ analogues of the present invention will not affect the efficacy of these analogues as GH antagonists. If some or all of these amino acids were added to the C-terminus of the hGH-RH(1-29)-NH₂ analogues, the added amino acid residues could be the same as residues 30 through 44 in the native hGH-RH sequence or reasonable equivalents.

Synthetic Methods.

The synthetic peptides are synthesized by a suitable method such as by exclusive solid phase techniques, by partial solid-phase techniques, by fragment condensation or by classical solution phase synthesis.

When the analogues of this invention are synthesized by solid-phase method, the C-terminus residue (here, R²⁹) is appropriately linked (anchored) to an inert solid support (resin) while bearing protecting groups for its alpha amino group (and, where appropriate, for its side chain functional group). After completion of this step, the alpha amino protecting group is removed from the anchored amino acid residue and the next amino acid residue, R²⁸, is added having its alpha amino group (as well as any appropriate side chain functional group) suitably protected, and so forth. The N-terminus protecting groups are removed after each residue is added, but the side chain protecting groups are not yet removed. After all the desired amino acids have been linked in the proper sequence, the peptide is cleaved from the support and freed from any side chain protecting group(s) under conditions that are minimally destructive towards residues in the sequence.

This is followed by a careful purification and scrupulous characterization of the synthetic product, so as to ensure that the desired structure is indeed the one obtained.

It is particularly preferred to protect the alpha amino function of the amino acids during the coupling step with an acid or base sensitive protecting group. Such protecting groups should have the properties of being stable in the conditions of peptide linkage formation, while being readily removable without destruction of the growing peptide chain and without racemization of any of the chiral centers contained therein. Suitable alpha amino protecting groups are Boc and Fmoc.

Medical Applications.

The hGH-RH antagonist peptides, or salts of these peptides, may be formulated in pharmaceutical dosage forms containing effective amounts thereof and administered to humans or animal for therapeutic or diagnostic purposes. The peptides may be used to suppress GH levels and to treat conditions associated with excessive levels of GH, e.g., diabetic retinopathy and nephropathy, and acromegaly. Also provided are methods for treating these diseases by administration of a composition of the invention to an individual needing such treatment. The main uses of GH-RH antagonists are however, in the field of cancer, for example human cancers of the breast, lung, colon, brain, and pancreas where the receptors for IGF-I are present.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 is a plot of tumor volumes in athymic nude mice bearing s.c. transplanted SK-ES-1 human sarcomas during treatment with Peptide 19 administered from osmotic minipumps at a dose of 40 μ g/animal/day. Treatment was started when the tumors measured approximately 33-39 mm³ and lasted for 4 and 3 weeks, respectively.

Figure 2 is a plot of tumor volumes in athymic nude mice bearing s.c. transplanted MNNG/HOS human sarcomas during treatment with Peptide 19 administered from osmotic minipumps at a dose of 40 μ g/animal/day.

Treatment was started when the tumors measured approximately 33-39 mm³ and lasted for 4 and 3 weeks, respectively.

Figure 3 is a plot of the inhibitory effect of GH-RH antagonist Peptide 19 on growth of MXT estrogen independent mouse mammary cancer.

5

DETAILED DESCRIPTION OF THE PREFERRED EMBODIMENTS

A. Abbreviations

The nomenclature used to define the peptides is that specified by the IUPAC-IUB Commissioner on Biochemical Nomenclature wherein, in accordance with conventional representation, the amino group at the N-terminus appears to the left and the carboxyl group at the C-terminus appears to the right. The term "natural amino acid" as used herein means one of the common, naturally occurring L-amino acids found in naturally occurring proteins: Gly, Ala, Val, Leu, Ile, Ser, Thr, Lys, Arg, Asp, Asn, Glu, Gln, Cys, Met Phe, Tyr, Pro, Trp and His. When the natural amino acid residue has isomeric forms, it is the L-form of the amino acid that is represented herein unless otherwise expressly indicated.

Non-coded amino acids, or amino acid analogues, are also incorporated into the GH-RH antagonists. ("Non-coded" amino acids are those amino acids which are not among the approximately 20 natural amino acids found in naturally occurring peptides.) Among the non-coded amino acids or amino acid analogues which may be used in the hGH-RH antagonist peptides are the following: by Abu is meant alpha amino butyric acid, by Agm is meant agmatine (1-amino-4-guanidino-butane), by Aib meant alpha amino isobutyric acid, by Har is meant homoarginine, by hPhe is meant homo-phenylalanine, by Nal is meant 2-naphthyl-alanine, and by Nle is meant norleucine. When these non-coded amino acids, or amino acid analogues, have isomeric forms, it is the L-form of the amino acid that is represented unless otherwise expressly indicated.

Abbreviations used herein are:

| | |
|---------------------|---|
| Abu | α -aminobutyric acid |
| Ac | acetyl |
| AcOH | acetic acid |
| 5 Ac ₂ O | acetic anhydride |
| Agm | agmatine (1-amino-4-guanidino-butane) |
| Aib | α -aminoisobutyric acid |
| Aqc | anthraquinone-2-carbonyl |
| BHA | benzhydramine |
| 10 Boc | tert.butyloxycarbonyl |
| Bom | benzyloxymethyl |
| BOP | benzotriazole-1-yl-oxy-tris-(dimethylamino)- phosphonium hexafluorophosphate |
| BrProp | bromopropionyl |
| 15 Bzl | benzyl |
| CHx | cyclohexyl |
| Cit | citrulline, i.e., 2-amino-5-ureidovaleric acid |
| DCC | dicyclohexylcarbodiimide |
| DCM | dichloromethane |
| 20 DIC | N,N'-diisopropylcarbodiimide |
| DIEA | diisopropylethylamine |
| DMF | dimethylformamide |
| Fmoc | fluorenylmethyloxycarbonyl |
| For | Formyl |
| 25 GH | growth hormone |
| GH-RH | GH releasing hormone |
| Glt | glutaryl |
| Har | homoarginine |
| hGH-RH | human GH-RH |
| 30 HOBt | 1-hydroxybenzotriazole |
| hPhe | homophenylalanine |

| | |
|-------------|--|
| HPLC | high performance liquid chromatography |
| IAC | iodoacetyl |
| Ibu | isobutyryl |
| MeOH | methanol |
| 5 MeCN | acetonitrile |
| MBHA | para-methylbenzhydramine |
| Nac | 1-naphthylacetyl |
| 2-Nac | 2-naphthylacetyl |
| NaI | 2-naphthyl-alanine |
| 10 Nle | norleucine |
| NMM | N-methylmorpholine |
| Npr | naphthylpropionyl |
| 1-Npt | 1-naphthoyl |
| 2-Npt | 2-naphthoyl |
| 15 Phe(pCl) | para-chloro-phenylalanine |
| rGH-RH | rat GH-RH |
| RP-HPLC | reversed phase HPLC |
| SPA | sulfophenoxy acetyl |
| TFA | trifluoroacetic acid |
| 20 Tos | para-toluenesulfonyl |
| Tpi | 2,3,4,9-tetrahydro-1H-pyrido[3,4-b]indol-3-carboxylic acid |
| Z | benzyloxycarbonyl |
| ϕ | unsubstituted aromatic ring |

25

B. The GH-RH Analogues

The hGH-RH analogues of the present invention were designed to increase the affinities of the peptides to the receptor, to improve metabolic stability and to maximize the amphophilic secondary structure of the molecules. Many of these analogues cause very effective and long lasting inhibition of GH release stimulated by hGH-RH(1-29)NH₂.

30

The following embodiments are specially preferred as having remarkable bioactivity:

| | | |
|----|--|--------------|
| | [Nac ⁰ ,D-Arg ² ,Phe(pCl) ⁶ ,Abu ¹⁵ ,Nle ²⁷]hGH-RH(1-29)NH ₂ | Peptide # 1 |
| | [Ac ⁰ -His ¹ ,D-Arg ² ,Phe(pCl) ⁶ ,Abu ¹⁵ ,Nle ²⁷]hGH-RH(1-29)NH ₂ | Peptide # 2 |
| 5 | [Ibu ⁰ -His ¹ ,D-Arg ² ,Phe(pCl) ⁶ ,Abu ¹⁵ ,Nle ²⁷]hGH-RH(1-29)NH ₂ | Peptide # 3 |
| | [IAc ⁰ -His ¹ ,D-Arg ² ,Phe(pCl) ⁶ ,Abu ¹⁵ ,Nle ²⁷]hGH-RH(1-29)NH ₂ | Peptide # 4 |
| | [Nac ⁰ -His ¹ ,D-Arg ² ,Phe(pCl) ⁶ ,Abu ¹⁵ ,Nle ²⁷]hGH-RH(1-29)NH ₂ | Peptide # 5 |
| | [Glt ¹ ,D-Arg ² ,Phe(pCl) ⁶ ,Abu ¹⁵ ,Nle ²⁷]hGH-RH(1-29)NH ₂ | Peptide # 6 |
| | [Ibu ⁰ -Glu ¹ ,D-Arg ² ,Phe(pCl) ⁶ ,Abu ¹⁵ ,Nle ²⁷]hGH-RH(1-29)NH ₂ | Peptide # 7 |
| 10 | [IAc ⁰ -Glu ¹ ,D-Arg ² ,Phe(pCl) ⁶ ,Abu ¹⁵ ,Nle ²⁷]hGH-RH(1-29)NH ₂ | Peptide # 8 |
| | [Nac ⁰ -Glu ¹ ,D-Arg ² ,Phe(pCl) ⁶ ,Abu ¹⁵ ,Nle ²⁷]hGH-RH(1-29)NH ₂ | Peptide # 9 |
| | [Ibu ⁰ -His ¹ ,D-Arg ² ,Tpi ⁶ ,Abu ¹⁵ ,Nle ²⁷]hGH-RH(1-29)NH ₂ | Peptide # 10 |
| | [IAc ⁰ -His ¹ ,D-Arg ² ,Tpi ⁶ ,Abu ¹⁵ ,Nle ²⁷]hGH-RH(1-29)NH ₂ | Peptide # 11 |
| | [Glt ¹ ,D-Arg ² ,Tpi ⁶ ,Abu ¹⁵ ,Nle ²⁷]hGH-RH(1-29)NH ₂ | Peptide # 12 |
| 15 | [Ibu ⁰ ,D-Arg ² ,Aib ⁸ ,Abu ¹⁵ ,Nle ²⁷]hGH-RH(1-29)NH ₂ | Peptide # 13 |
| | [Ibu ⁰ ,D-Arg ² ,Phe(pCl) ⁶ ,Aib ⁸ ,Abu ¹⁵ ,Nle ²⁷]hGH-RH(1-29)NH ₂ | Peptide # 14 |
| | [Ibu ⁰ ,D-Arg ² ,Phe(pCl) ⁶ ,Ala ¹² ,Abu ¹⁵ ,Nle ²⁷]hGH-RH(1-29)NH ₂ | Peptide # 15 |
| | [Ibu ⁰ ,D-Arg ² ,Phe(pCl) ⁶ ,Abu ^{15,19} ,Nle ²⁷]hGH-RH(1-29)NH ₂ | Peptide # 16 |
| | [Ibu ⁰ -Glu ¹ ,D-Arg ² ,Phe(pCl) ⁶ ,Abu ^{15,19} ,Nle ²⁷]hGH-RH(1-29)NH ₂ | Peptide # 17 |
| 20 | [Nac ⁰ ,D-Arg ² ,Phe(pCl) ⁶ ,Abu ¹⁵ ,Nle ²⁷]hGH-RH(1-28)Agm | Peptide # 18 |
| | [Ibu ⁰ ,D-Arg ² ,Phe(pCl) ⁶ ,Abu ¹⁵ ,Nle ²⁷]hGH-RH(1-28)Agm | Peptide # 19 |
| | [BrProp ⁰ ,D-Arg ² ,Phe(pCl) ⁶ ,Abu ¹⁵ ,Nle ²⁷]hGH-RH(1-28)Agm | Peptide # 20 |
| | [IAc ⁰ ,D-Arg ² ,Phe(pCl) ⁶ ,Abu ¹⁵ ,Nle ²⁷]hGH-RH(1-28)Agm | Peptide # 21 |
| | [Nac ⁰ -His ¹ ,D-Arg ² ,Phe(pCl) ⁶ ,Abu ¹⁵ ,Nle ²⁷ ,Asp ²⁸]hGH-RH(1-28)Agm | Peptide # 22 |
| 25 | [Nac ⁰ ,D-Arg ² ,Phe(pCl) ⁶ ,Abu ¹⁵ ,Nle ²⁷ ,Asp ²⁸]hGH-RH(1-28)Agm | Peptide # 23 |
| | [2-Nac ⁰ ,D-Arg ² ,Phe(pCl) ⁶ ,Abu ¹⁵ ,Nle ²⁷ ,Asp ²⁸]hGH-RH(1-28)Agm | Peptide # 24 |
| | [1-Npt ⁰ ,D-Arg ² ,Phe(pCl) ⁶ ,Abu ¹⁵ ,Nle ²⁷ ,Asp ²⁸]hGH-RH(1-28)Agm | Peptide # 25 |
| | [Aqc ⁰ ,D-Arg ² ,Phe(pCl) ⁶ ,Abu ¹⁵ ,Nle ²⁷ ,Asp ²⁸]hGH-RH(1-28)Agm | Peptide # 26 |
| | [Nac ⁰ ,D-Arg ² ,Phe(pCl) ⁶ ,Ala ¹⁵ ,Nle ²⁷]hGH-RH(1-28)Agm | Peptide # 27 |
| 30 | [Nac ⁰ ,D-Arg ² ,Gly ³ ,Phe(pCl) ⁶ ,Abu ¹⁵ ,Nle ²⁷ ,Asp ²⁸]hGH-RH(1-28)Agm | Peptide # 28 |
| | [IAc ⁰ ,D-Arg ² ,Pro ⁶ ,Abu ¹⁵ ,Nle ²⁷ ,Asp ²⁸]hGH-RH(1-28)Agm | Peptide # 29 |
| | [Ibu ⁰ ,D-Arg ² ,Pro ⁶ ,Abu ¹⁵ ,Nle ²⁷ ,Asp ²⁸]hGH-RH(1-28)Agm | Peptide # 30 |
| | [IAc ⁰ ,D-Arg ² ,hPhe ⁶ ,Abu ¹⁵ ,Nle ²⁷ ,Asp ²⁸]hGH-RH(1-28)Agm | Peptide # 31 |

| | | |
|---|---|--------------|
| | [Nac ⁰ ,D-Arg ² ,Nal ⁶ ,Abu ¹⁵ ,Nle ²⁷]hGH-RH(1-28)Agm | Peptide # 32 |
| | [Nac ⁰ ,D-Arg ² ,Ala ⁶ ,Abu ¹⁵ ,Nle ²⁷ ,Asp ²⁸]hGH-RH(1-28)Agm | Peptide # 33 |
| | [Nac ⁰ ,D-Cit ² ,Phe(pCl) ⁶ ,Abu ¹⁵ ,Nle ²⁷]hGH-RH(1-28)Agm | Peptide # 34 |
| | [D-Cit ² ,Phe(pCl) ⁶ ,Abu ¹⁵ ,Nle ²⁷]hGH-RH(1-28)Agm | Peptide # 35 |
| 5 | [Nac ⁰ ,D-Cit ² ,Nal ⁶ ,Abu ¹⁵ ,Nle ²⁷]hGH-RH(1-28)Agm | Peptide # 36 |
| | [D-Arg ² ,Phe(pCl) ⁶ ,Abu ¹⁵ ,Nle ²⁷]hGH-RH(1-28)Agm | Peptide # 37 |
| | [For ⁰ ,D-Arg ² ,Phe(pCl) ⁶ ,Abu ¹⁵ ,Nle ²⁷]hGH-RH(1-28)Agm | Peptide # 38 |

Three highly preferred embodiments have the following formulae:

| | | |
|----|---|--------------|
| 10 | [Nac ⁰ ,D-Arg ² ,Phe(pCl) ⁶ ,Abu ¹⁵ ,Nle ²⁷]hGH-RH(1-28)Agm | Peptide # 18 |
| | [Nac ⁰ ,D-Arg ² ,Nal ⁶ ,Abu ¹⁵ ,Nle ²⁷]hGH-RH(1-28)Agm | Peptide # 32 |
| | [Nac ⁰ ,D-Cit ² ,Phe(pCl) ⁶ ,Abu ¹⁵ ,Nle ²⁷]hGH-RH(1-28)Agm | Peptide # 34 |

The most preferred embodiments have the following formulae:

| | | |
|----|---|--------------|
| 15 | [Nac ⁰ ,D-Arg ² ,Phe(pCl) ⁶ ,Abu ¹⁵ ,Nle ²⁷]hGH-RH(1-29)NH ₂ | Peptide # 1 |
| | [Nac ⁰ -His ¹ -D-Arg ² ,Phe(pCl) ⁶ ,Abu ¹⁵ ,Nle ²⁷]hGH-RH(1-29)NH ₂ | Peptide # 5 |
| | [Ibu ⁰ ,D-Arg ² ,Phe(pCl) ⁶ ,Abu ¹⁵ ,Nle ²⁷]hGH-RH(1-28)Agm | Peptide # 19 |
| | [For ⁰ ,D-Arg ² ,Phe(pCl) ⁶ ,Abu ¹⁵ ,Nle ²⁷]hGH-RH(1-28)Agm | Peptide # 38 |

20 C. Method of Preparation

1. Overview of Synthesis.

The peptides are synthesized by a suitable method such as by exclusive solid phase techniques, by partial solid-phase techniques, by fragment condensation or by classical solution phase synthesis. For example, the techniques of exclusive solid-phase synthesis are set forth in the textbook "Solid Phase Peptide Synthesis", J.M. Stewart and J.D. Young, Pierce Chem. Company, Rockford, 111, 1984 (2nd. ed.), and M. Bodanszky, "Principles of Peptide Synthesis", SpringerVerlag, 1984. The hGH-RH antagonist peptides are preferably prepared using solid phase synthesis, such as that generally described by Merrifield, J.Am.Chem.Soc., 85, p. 2149 (1963), although other equivalent chemical syntheses known in the art can also be used as previously mentioned.

The synthesis is carried out with amino acids that are protected at their alpha amino group. Urethane type protecting groups (Boc or Fmoc) are preferably used for the protection of the alpha amino group. The preferred protecting group is Boc.

5

In solid phase synthesis, the moiety which forms the aminoacyl group of the final peptide at the C-terminus is attached to a polymeric resin support via a chemical link. After completion of the coupling reaction, the alpha amino protecting group is selectively removed to allow subsequent
10 coupling reactions to take place at the amino-terminus, preferably with 50% TFA in DCM. The remaining amino acids with similarly Boc-protected alpha amino groups are coupled stepwise to the free amino group of the preceding amino acid on the resin to obtain the desired peptide sequence. Because the amino acid residues are added to the alpha amino group of the C-
15 terminus residue, growth of the synthetic hGH-RH analogue peptides begins at the C terminus and progresses toward the N-terminus. When the desired sequence has been obtained, the peptide is acylated, if appropriate, and it is removed from the support polymer.

20 Each protected amino acid is used in excess (2.5 or 3 equivalents) and the coupling reactions are usually carried out in DCM, DMF or mixtures thereof. The extent of completion of the coupling reaction is monitored at each stage by the ninhydrin reaction. In cases where incomplete coupling is determined, the coupling procedure is repeated before removal of the
25 alpha amino protecting group prior to the coupling of the next amino acid.

A typical synthesis cycle is shown in Table I.

TABLE I
Protocol for a Typical Synthetic Cycle Using Boc-strategy

| Step 5 (min) | Reagent | Mixing Time |
|--------------------|--|-------------|
| <hr/> | | |
| 1. Deprotection | 50% TFA in DCM | |
| 5 + 25 | | |
| | DCM wash | 1 |
| 10 | 2-propanol wash | 1 |
| 2. Neutralization | 5% DIEA in DCM | 1 |
| | DCM wash | 1 |
| | MeOH wash | 1 |
| | 5% DIEA in DCM | 3 |
| 15 | MeOH wash | 1 |
| | DCM wash (3 times) | 1-1 |
| 3. Coupling | 3 equiv. Boc-amino acid in DCM or DMF + 3 equiv. DIC or the preformed HOBT ester of the Boc-amino acid | 60 |
| 20 | MeOH wash | 2 |
| | DCM wash | 2 |
| | MeOH wash | 2 |
| | DCM wash | 2 |
| | MeOH wash | 2 |
| 25 | DCM wash | 2 |
| 4. Acetylation | Ac ₂ O in DCM (30%) | 10 |
| + 20 | | |
| (if appropriate) | MeOH wash (3 times) | 2 |
| | DCM wash (3 times) | 2 |
| 30 | | |

After completion of the synthesis, the cleavage of the peptide from the resin can be effected using procedures well known in peptide chemistry.

Some of the amino acid residues of the peptides have side chain functional groups which are reactive with reagents used in coupling or deprotection. When such side chain groups are present, suitable protecting groups are joined to these functional groups to prevent undesirable chemical reactions from occurring during the reactions used to form the peptides. The following general rules are followed in selecting a particular side chain protecting group: (a) the protecting group preferably retains its protecting properties and is not split off under coupling conditions, (b) the protecting group should be stable to the reagent used in the coupling reaction conditions and in conditions for removing the alpha amino protecting group at each step of the synthesis and, (c) the side chain protecting group must be removable upon the completion of the synthesis of the desired amino acid sequence, under reaction conditions that will not undesirably alter the peptide chain.

15

The initial synthetic steps utilized herein are disclosed in US Patent 4,914,189 which is incorporated by reference herein. Reference is particularly made to Examples I through IV therein.

20

2. Coupling R²⁹ to the Support Polymer.

The hGH-RH antagonist peptides may be synthesized on a variety of support polymers. These support polymers may be amino resins such as amino-methyl resins, benzhydrylamine resins, p-methylbenzhydrylamine resins and the like. Boc-R²⁹ is the initial material joined to the support phase, suitably Boc-Arg(Tos)-OH or Boc-Agm.

25

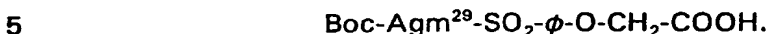
For the synthesis of peptides having Agm at the C-terminus, it is preferred that the support phase [SP] is an amino methyl resin. The guanidino group of Boc-Agm is joined to the support polymer via a stable but readily cleavable bridging group. It has been found that such a bridge may be readily provided by the sulfonyl phenoxy acetyl moiety. The alpha

30

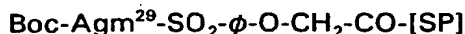
amino Boc-protected Agm is reacted with the chlorosulfonyl phenoxy acetic acid



to form



This compound is then coupled to the support polymer [SP] using DIC or BOP as activating reagent to yield:



10

For the synthesis of peptides having Arg-NH₂ at the C-terminus, Boc-Arg(Tos)-OH is coupled to the neutralized BHA or MBHA resin using DIC or BOP as activating reagent.

15 3. Stepwise Coupling of Amino Acid Residues.

Utilizing the Boc-protected Agm resin (California Peptide Res. Inc.), (or the Boc-Arg(Tos)-resin), the peptide itself may suitably be built up by solid phase synthesis in the conventional manner. The selection of an appropriate coupling reagent is within the skill of the art. Particularly
20 suitable as coupling reagents are N,N'-diisopropyl carbodiimide (DIC) or the BOP carboxyl activating reagent.

Each protected amino acid is coupled in about a three-fold molar excess, with respect to resin-bound aminoacyl residue(s), and the coupling
25 may be carried out in as medium such as DMF: CH₂Cl₂ (1:1) or in DMF or CH₂Cl₂ alone. In cases where incomplete coupling occurs, the coupling procedure is repeated before removal of the alpha amino protecting group. The success of the coupling reaction at each stage of the synthesis is preferably monitored by the ninhydrin reaction.

30

4. Removal of the Peptide from the Support Polymer.

When the synthesis is complete, the peptide is cleaved from the support phase. Removal of the peptide from the resin is performed by treatment with a reagent such as liquid hydrogen fluoride which also cleaves
5 all remaining side chain protecting groups.

Suitably, the dried and protected peptide-resin is treated with a mixture consisting of 1.0 mL m-cresol and 10 mL anhydrous hydrogen fluoride per gram of peptide-resin for 60 min at 0°C to cleave the peptide
10 from the resin as well as to remove all side chain protecting groups. After the removal of the hydrogen fluoride under a stream of nitrogen and vacuum, the free peptides are precipitated with ether, filtered, washed with ether and ethyl acetate, extracted with 50% acetic acid, and lyophilized.

5. Purification

The purification of the crude peptides can be effected using procedures well known in peptide chemistry. For example, purification may be performed on a MacRabbit HPLC system (Rainin Instrument Co. Inc., Woburn, MA) with a Knauer UV Photometer and a Kipp and Zonen BD40
20 Recorder using a 10 x 250 mm VYDAC 228TP column packed with C8 silica gel (300 Å pore size, 10 µm particle size) (Rainin Inc.). The column is eluted with a solvent system consisting of (A) 0.1% aqueous TFA and (B) 0.1% TFA in 70% aqueous MeCN in a linear gradient mode (e.g., 30-65% B in 120 min). The eluent is monitored at 220 nm, and fractions are
25 examined by analytical HPLC using a Hewlett-Packard Model HP-1090 liquid chromatograph and pooled to give maximum purity. Analytical HPLC is carried out on a W-Porex C18 reversed-phase column (4.6 x 250 mm, 5 µm particle size, 300 Å pore size) (Phenomenex, Rancho Palos Verdes, CA) using isocratic elution with a solvent system consisting of (A) and (B)
30 defined above. The peaks are monitored at 220 and 280 nm. The peptides

are judged to be substantially (>95%) pure by analytical HPLC. The expected amino acid composition is also confirmed by amino acid analysis.

D. Pharmaceutical Composition

5 The peptides of the invention may be administered in the form of pharmaceutically acceptable, nontoxic salts, such as acid addition salts. Illustrative of such acid addition salts are hydrochloride, hydrobromide, sulphate, phosphate, fumarate, gluconate, tannate, maleate, acetate, citrate, benzoate, succinate, alginate, pamoate, malate, ascorbate, tartarate,
10 and the like. Particularly preferred antagonists are salts of low solubility, e.g., pamoate salts and the like. These exhibit long duration of activity.

 The compounds of the present invention are suitably administered to subject humans or animals s.c., i.m., or i.v; intranasally or by pulmonary
15 inhalation; or in a depot form (e.g., microcapsules, microgranules, or cylindrical rod like implants) formulated from a biodegradable suitable polymer (such as D,L-lactide-coglycolide), the former two depot modes being preferred. Other equivalent modes of administration are also within the scope of this invention, i.e., continuous drip, depot injections, infusion
20 pump and time release modes such as microcapsules and the like. Administration is in any physiologically acceptable injectable carrier, physiological saline being acceptable, though other carriers known to the art may also be used.

25 The peptides are preferably administered parenterally, intramuscularly, subcutaneously or intravenously with a pharmaceutically acceptable carrier such as isotonic saline. Alternatively, the peptides may be administered as an intranasal spray with an appropriate carrier or by pulmonary inhalation. One suitable route of administration is a depot form formulated from a
30 biodegradable suitable polymer, e.g., poly-D,L-lactide-coglycolide as

microcapsules, microgranules or cylindrical implants containing dispersed antagonistic compounds.

The amount of peptide needed depends on the mode of
5 administration and the intended result. In general, the dosage range is between 1-100 $\mu\text{g/kg}$ of body weight of the host per day.

E. Therapeutic Uses of GH-RH Antagonists

hGH-RH antagonists can be used in treatment of conditions caused
10 by excess growth hormone, for example acromegaly, which is manifested by an abnormal enlargement of the bones of the face and extremities. The GH-RH antagonists may also be used to treat diabetic retinopathy (the main cause of blindness in diabetics) and diabetic retinopathy, in which damage to the eye and kidney respectively is thought to be due to GH.

15

The hGH-RH antagonists are designed to block the binding and therefore the action of GH-RH, which stimulates the secretion of GH, which in turn stimulates production of IGF I. GH-RH antagonists may be administered alone or together with somatostatin analogues, a combination
20 which more completely suppresses IGF-I levels. It is advantageous to administer antagonists of GH-RH rather than somatostatin due to the fact that GH-RH antagonists may be utilized in situations where target sites do not have somatostatin receptors.

25 However, the main applications of GH-RH antagonists are in the field of cancer. This is based on the following considerations: GH-RH antagonists are designed to block the binding and therefore the action of GH-RH, which stimulates the secretion of GH, which in turn stimulates production of insulin-like growth factor I (IGF-I) also called somatomedin-C.
30 The involvement of IGF-I (somatomedin-C) in breast cancer, prostate cancer, colon cancer, bone tumors and other malignancies is well established, and

somatostatin analogues alone do not adequately suppress GH and IGF-I levels. A complete suppression of IGF-I levels or secretion is required for a better inhibition of tumor growth. Autocrine production of IGF-I by various tumors could be also under control of GH-RH and might therefore be
5 inhibited by GH-RH antagonists. GH-RH antagonists might also inhibit the production of IGF-I. A more detailed theoretical background of the applications of GH-RH in the field of oncology (cancer) is as follows: The receptors for IGF-I are present in primary human breast cancers, in lung cancers, in human colon cancers, in human brain tumors, and in human
10 pancreatic cancers.

The presence of IGF-I receptors in these tumors appears to be related to malignant transformation and proliferations of these cancers. IGF-I can act as endocrine, paracrine or autocrine growth factor for various human
15 cancers, that is the growth of these neoplasms is dependent on IGF-I. GH-RH antagonists by suppressing GH secretion would lower the production of IGF-I. Since IGF-I stimulates growth of these various neoplasms (cancers), the lowering of circulating IGF-I levels should lead to tumor growth inhibition. It is possible that GH-RH antagonists could also lower paracrine
20 or autocrine production of IGF-I by the tumors, which should also lead to inhibition of cancer proliferation. These views are in accordance with modern concepts of clinical oncology. GH-RH antagonists should be given alone or together with somatostatin analogues and a combination would achieve a more complete suppression of IGF-I levels, elimination of tissue
25 IGF-I levels, e.g., in human osteosarcomas, as well as breast cancer, colon cancer, prostate cancer, and non-small cell lung cancer (non-SCLC).

The advantage of GH-RH antagonists over somatostatin analogues is based on the fact that GH-RH antagonists may be utilized for suppression
30 of tumors which do not have somatostatin receptors, for example human osteogenic sarcomas.

The present invention is described in connection with the following examples which are set forth for the purposes of illustration only.

- 5 The following Examples set forth suitable methods of synthesizing the novel GH-RH antagonists by the solid-phase technique.

EXAMPLE I

Synthesis of Boc-agmatine

EXAMPLE II

- 10 *Synthesis of 4-Chlorosulfonyl Phenoxyacetic Acid (Cl-SPA)*

EXAMPLE III

Boc-agmatine-[SPA]

EXAMPLE IV

Coupling of Boc-agmatine-[SPA] to Support Phase

- 15 The initial synthetic sequence utilized herein and indicated by headings above is disclosed in Examples I through IV of US Patent 4,914,189, which Examples are incorporated herein by reference.

EXAMPLE V

- 20 The synthesis of Peptide 1 having the formula:
Nac⁰-Tyr¹-D-Arg²-Asp³-Ala⁴-Ile⁵-Phe(pCl)⁶-Thr⁷-Asn⁸-Ser⁹-Tyr¹⁰-Arg¹¹-Lys¹²-
Val¹³-Leu¹⁴-Abu¹⁵-Gln¹⁶-Leu¹⁷-Ser¹⁸-Ala¹⁹-Arg²⁰-Lys²¹-Leu²²-Leu²³-Gln²⁴-Asp²⁵-
Ile²⁶-Nle²⁷-Ser²⁸-Arg²⁹-NH₂

or [Nac⁰,D-Arg²,Phe(pCl)⁶,Abu¹⁵,Nle²⁷]hGH-RH(1-29)NH₂

- 25 is conducted in a stepwise manner using manual solid phase peptide synthesis equipment. Briefly, 4-methyl-benzhydrylamine (MBHA) resin (Bachem, California) (200 mg, 0.11 mmole) is neutralized with 5% DIEA in CH₂Cl₂ and washed according to the protocol described in Table I. The solution of Boc-Arg(Tos)-OH (141 mg, 0.33 mmole) in DMF-CH₂Cl₂ (1:1) is
30 shaken with the neutralized resin and DIC (57 μ L, 0.36 mmole) in a manual solid phase peptide synthesis equipment for 1 hour. After the completion

of the coupling reaction is proved by negative ninhydrin test, deprotection with 50% TFA in CH_2Cl_2 , and neutralization with 5% DIEA, the peptide chain is built stepwise by adding the following protected amino acids in the indicated order on the resin to obtain the desired peptide sequence:

- 5 Boc-Ser(Bzl)-OH, Boc-Nle-OH, Boc-Ile-OH, Boc-Asp(OcHx)-OH, Boc-Gln-OH, Boc-Leu-OH, Boc-Leu-OH, Boc-Lys(2-Cl-Z)-OH, Boc-Arg(Tos)OH, Boc-Ala-OH, Boc-Ser(Bzl)-OH, Boc-Leu-OH, Boc-Gln-OH, Boc-Abu-OH, Boc-Leu-OH, Boc-Val-OH, Boc-Lys(2-Cl-Z)-OH, Boc-Arg(Tos)OH, Boc-Tyr(2,6-diCl-Z)-OH, Boc-Ser(Bzl)-OH, Boc-Asn-OH, Boc-Thr(Bzl)-OH, Boc-Phe(pCl)-OH, Boc-Ile-
10 OH, Boc-Ala-OH, Boc-Asp(OcHx)-OH, Boc-D-Arg(Tos)OH, and Boc-Tyr(2,6-diCl-Z)-OH.

These protected amino acid residues (also commonly available from Bachem Co.) are represented above according to a well accepted convention. The suitable protecting group for the side chain functional group of particular amino acids appears in parentheses. The OH groups in the above formulae indicate that each residue's carboxyl terminus is free.

The protected amino acids (0.33 mmole each) are coupled with DIC (57 μL , 0.36 mmole), with the exceptions of Boc-Asn-OH and Boc-Gln-OH which are coupled with their preformed HOBt esters. After removal of the Boc protecting group from the alpha amino group of Tyr¹, the alpha amino group of Tyr¹ is acylated. This is performed by the symmetrical anhydride method, in which 1-naphthylacetic acid (123 mg, 0.66 mmole) is reacted with DIC as an activating agent (60 μL , 0.37 mmole) to form a symmetric anhydride of 1-naphthylacetic acid. This symmetrical anhydride is reacted with the peptide.

In order to cleave the peptide from the resin and deprotect it, the dried peptide resin (325 mg) is stirred with 0.5 mL m-cresol and 5 mL hydrogen fluoride (HF) at 0°C for 1 hour. After evaporation of the HF under

vacuum, the remnant is washed with dry diethyl ether and ethyl acetate. The cleaved and deprotected peptide is dissolved in 50 % acetic acid and separated from the resin by filtration. After dilution with water and lyophilization, 145 mg crude product is obtained.

5

The crude peptide is checked by analytical HPLC using a Hewlett-Packard Model HP-1090 liquid chromatograph with a W-Porex C18 reversed-phase column (4.6 x 250mm, 5 μ m particle size, 300 Å pore size from Phenomenex, Rancho Palos Verdes, CA) and linear gradient elution, (e.g., 35-70% B) with a solvent system consisting of (A) 0.1% aqueous TFA and (B) 0.1% TFA in 70% aqueous MeCN. 60 mg of the crude peptide is dissolved in AcOH/H₂O), stirred, filtered and applied on a VYDAC 228TP column (10 x 250 mm) packed with C8 silica gel. The column is eluted with a solvent system described above in a linear gradient mode (e.g., 30-55% B in 120 min); flow rate 3mL/min. The eluent is monitored at 220 nm, and fractions are examined by analytical HPLC. Fractions with purity higher than 95% are pooled and lyophilized to give 3.5 mg pure product. The analytical HPLC is carried out on a W-Porex C18 reversed-phase column described above using isocratic elution with a solvent system described above with a flow rate of 1.2 mL/min. The peaks are monitored at 220 and 280 nm. R_t = 13.70 min and k' = 0.828 (isocratic elution with 52% B). The peptides are judged to be substantially (>95%) pure by analytical HPLC. The expected amino acid composition is also confirmed by amino acid analysis.

25

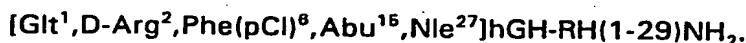
Peptides 2, 3, 4 and 5 are synthesized in the same manner as Peptide 1, except that Boc-Tyr(2,6-diCl-Z)-OH¹ is replaced with Boc-His(Bom)-OH¹ (0.33 mmole) and the resulting peptides are acylated with the appropriate anhydrides of acetic acid, isobutyric acid, iodoacetic acid or 1-naphthyl-acetic acid respectively, to yield:

30

| | |
|---|-----------|
| [Ac ⁰ -His ¹ ,D-Arg ² ,Phe(pCl) ⁶ ,Abu ¹⁵ ,Nle ²⁷]hGH-RH(1-29)NH ₂ | Peptide 2 |
| [Ibu ⁰ -His ¹ ,D-Arg ² ,Phe(pCl) ⁶ ,Abu ¹⁵ ,Nle ²⁷]hGH-RH(1-29)NH ₂ | Peptide 3 |
| [IAc ⁰ -His ¹ ,D-Arg ² ,Phe(pCl) ⁶ ,Abu ¹⁵ ,Nle ²⁷]hGH-RH(1-29)NH ₂ | Peptide 4 |
| [Nac ⁰ -His ¹ ,D-Arg ² ,Phe(pCl) ⁶ ,Abu ¹⁵ ,Nle ²⁷]hGH-RH(1-29)NH ₂ | Peptide 5 |

5

Peptide 6 is synthesized in the same manner as Peptide 1, except that Boc-Tyr(2,6-diCl-Z)-OH¹ is omitted, and the final peptide's N-terminus D-Arg is acylated with glutaric anhydride to yield:



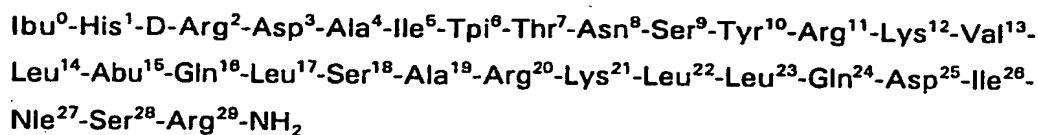
10

Peptides 7, 8 and 9 are synthesized in the same manner as Peptide 1 except that Boc-Tyr(2,6-diCl-Z)-OH¹ is replaced by Boc-Glu(OcHx)-OH¹ (0.33 mmole) and is acylated with the appropriate anhydride of isobutyric acid, iodoacetic acid and 1-naphthylacetic acid respectively, to yield:

| | |
|--|-----------|
| 15 [Ibu ⁰ -Glu ¹ ,D-Arg ² ,Phe(pCl) ⁶ ,Abu ¹⁵ ,Nle ²⁷]hGH-RH(1-29)NH ₂ | Peptide 7 |
| [IAc ⁰ -Glu ¹ ,D-Arg ² ,Phe(pCl) ⁶ ,Abu ¹⁵ ,Nle ²⁷]hGH-RH(1-29)NH ₂ | Peptide 8 |
| [Nac ⁰ -Glu ¹ ,D-Arg ² ,Phe(pCl) ⁶ ,Abu ¹⁵ ,Nle ²⁷]hGH-RH(1-29)NH ₂ | Peptide 9 |

EXAMPLE VI

20 The synthesis of Peptide 10 having the formula:



or [Ibu⁰-His¹,D-Arg²,Tpi⁶,Abu¹⁵,Nle²⁷]hGH-RH(1-29)NH₂ is conducted in a
 25 stepwise manner using manual solid phase peptide synthesis equipment. Benzhydrylamine (BHA) resin (Bachem, California) (200 mg, 0.11 mmole) is neutralized with 5 % DIEA in CH₂Cl₂ and washed according to the protocol described in Table I. The solution of Boc-Arg(Tos)-OH (141 mg, 0.33 mmole) in CH₂Cl₂-DMF (1:1) is shaken with the neutralized resin and
 30 DIC (60 μL, 0.37 mmole) in a manual solid phase peptide synthesis equipment for 1 hour. After the coupling reaction is proved to be complete

by negative ninhydrin test, deprotection with 50% TFA in CH_2Cl_2 , and neutralization with 5% DIEA in CH_2Cl_2 , the peptide chain is built by stepwise addition of the following protected amino acids in the indicated order on the resin to obtain the desired peptide sequence:

- 5 Boc-Ser(Bzl)-OH, Boc-Nle-OH, Boc-Ile-OH, Boc-Asp(OcHx)-OH, Boc-Gln-OH, Boc-Leu-OH, Boc-Leu-OH, Boc-Lys(2-Cl-Z)-OH, Boc-Arg(Tos)OH, Boc-Ala-OH, Boc-Ser(Bzl)-OH, Boc-Leu-OH, Boc-Gln-OH, Boc-Abu-OH, Boc-Leu-OH, Boc-Val-OH, Boc-Lys(2-Cl-Z)-OH, Boc-Arg(Tos)OH, Boc-Tyr(2,6-diCl-Z)-OH, Boc-Ser(Bzl)-OH, Boc-Asn-OH, Boc-Thr(Bzl)-OH, Boc-Tpi-OH, Boc-Ile-OH,
10 Boc-Ala-OH, Boc-Asp(OcHx)-OH, Boc-D-Arg(Tos)OH, and Boc-His(Bom)-OH.

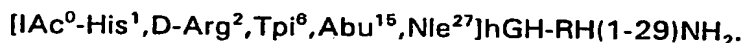
The protected amino acids (0.33 mmole each) are coupled with DIC (57 μL , 0.36 mmole) with the exceptions of Boc-Asn-OH and Boc-Gln-OH which are coupled with their preformed HOBt esters and Boc-Tpi-OH which
15 was coupled by using BOP coupling method. After removal of the Boc protecting group from the alpha amino group of His¹, the peptide is acylated using the symmetrical anhydride method. This is performed by reacting isobutyric acid (59 mg, 0.66 mmole) with DIC (60 μL , 0.37 mmole) to form the symmetrical anhydride thereof, and reacting this anhydride with the
20 peptide.

In order to cleave the peptide from the resin and deprotect it, the dried peptide resin (300-350 mg) is stirred with 0.5 mL m-cresol and 5 mL hydrogen fluoride (HF) at 0°C for 1 hour. After evaporation of the HF under
25 vacuum, the remnant is washed with dry diethyl ether and ethyl acetate. The cleaved and deprotected peptide is dissolved in 50% acetic acid and separated from the resin by filtration. After dilution with water and lyophilization, approximately 150 mg crude product is obtained.

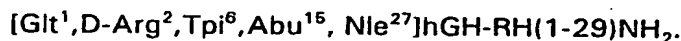
30 The crude peptide is purified (60 mg of the substance being purified by RP-HPLC using the same procedure and equipments described in Example

V), then checked by analytical HPLC. The product is judged to be substantially (>95%) pure by analytical HPLC. Confirmation of the structure is provided by amino acid analysis.

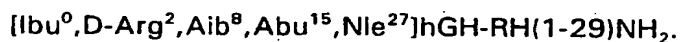
- 5 Peptide 11 is synthesized in the same manner as Peptide 10, except it is acylated with the appropriate anhydride of iodoacetic acid in place of isobutyric acid, to yield:



- 10 Peptide 12 is synthesized in the same manner as Peptide 10 except that Boc-His(Bom)-OH¹ and Ibu⁰ are omitted. The final peptide's N-terminus D-Arg is acylated with glutaric anhydride to yield:

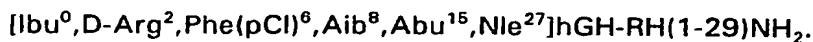


- 15 Peptide 13 is synthesized in the same manner as Peptide 10 except that Boc-His(Bom)-OH¹ is replaced with Boc-Tyr(2,6-diCl-Z)-OH¹; Boc-Tpi-OH⁶ is replaced with Boc-Phe-OH⁶; and Boc-Asn-OH⁸ is replaced with Boc-Aib-OH⁸, to yield:



20

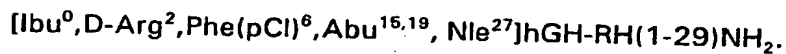
- Peptide 14 is synthesized in the same manner as Peptide 13, except that Boc-Phe-OH⁶ is replaced with Boc-Phe(pCl)-OH⁶ to yield:



- 25 Peptide 15 is synthesized in the same manner as Peptide 14 except that Boc-Aib-OH⁸ is replaced with Boc-Asn-OH⁸ and Boc-Lys(2-Cl-Z)-OH¹² is replaced with Boc-Ala-OH¹² to yield:



Peptide 16 is synthesized in the same manner as Peptide 15 except that Boc-Ala-OH¹² is replaced with Boc-Lys(2-Cl-Z)-OH¹² and Boc-Ala¹⁹-OH is replaced with Boc-Abu-OH to yield:



5

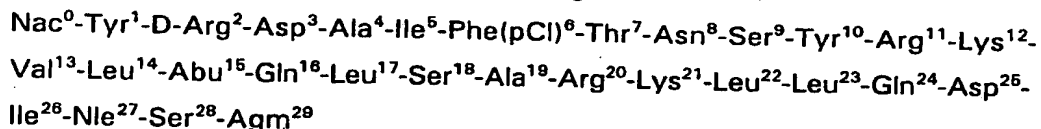
Peptide 17 is synthesized in the same manner as Peptide 16 except that Boc-Tyr(2,6-diCl-Z)-OH¹ is replaced with Boc-Glu(OcHx)-OH¹, to yield:

[Ibu⁰-Glu¹, D-Arg², Phe(pCl)⁶, Abu^{15,19}, Nle²⁷]hGH-RH(1-29)NH₂.

10

EXAMPLE VII

The synthesis of Peptide 18 having the formula:



15 or [Nac⁰, D-Arg², Phe(pCl)⁶, Abu¹⁵, Nle²⁷]hGH-RH(1-28)Agm, is conducted in a stepwise manner using manual solid phase peptide synthesis equipment.

Boc-Agm-SPA-aminomethyl resin (California Peptide Co., Inc., California) (200 mg, 0.06 mmole) is deprotected with 50% TFA in CH₂Cl₂,
 20 neutralized with 5% DIEA in CH₂Cl₂, and washed as described in Table I. A solution of Boc-Ser(Bzl)-OH (55 mg, 0.18 mmole) in CH₂Cl₂ is shaken with the H-Agm-SPA-aminomethyl resin and DIC (31 μ L, 0.2 mmole) in a manual solid phase peptide synthesis equipment for 1 hour. After wash and performance of the ninhydrin reaction to check for completeness of
 25 coupling, the cycle is repeated in a manner as described in Table I to build the peptide chain step-wise by adding the following protected amino acids in the indicated order on the resin:

Boc-Nle-OH, Boc-Ile-OH, Boc-Asp(OcHx)-OH, Boc-Gln-OH, Boc-Leu-OH, Boc-Leu-OH, Boc-Lys(2-Cl-Z)-OH, Boc-Arg(Tos)OH, Boc-Ala-OH, Boc-Ser(Bzl)-
 30 OH, Boc-Leu-OH, Boc-Gln-OH, Boc-Abu-OH, Boc-Leu-OH, Boc-Val-OH, Boc-Lys(2-Cl-Z)-OH, Boc-Arg(Tos)OH, Boc-Tyr(2,6-diCl-Z)-OH, Boc-Ser(Bzl)-OH,

Boc-Asn-OH, Boc-Thr(Bzl)-OH, Boc-Phe(pCl)-OH, Boc-Ile-OH, Boc-Ala-OH, Boc-Asp(OcHx)-OH, Boc-D-Arg(Tos)OH, and Boc-Tyr(2,6-diCl-Z)-OH.

The protected amino acids (0.18 mmole each) are coupled with DIC
5 (31 μ L, 0.2 mmole) with the exceptions of Boc-Asn-OH and Boc-Gln-OH
which are coupled with their preformed HOBt esters. After removal of the
Boc protecting group from the alpha amino group of Tyr¹, the peptide is
acylated by the symmetrical anhydride method. In this method, the
symmetrical anhydride of 1-naphthylacetic acid is formed by reacting 123
10 mg (or 0.66 mmole) of 1-naphthylacetic acid with 60 μ l (0.37 mmole) DIC;
the resulting symmetrical anhydride is reacted with the peptide.

In order to cleave the peptide from the resin and deprotect it, the
dried peptide resin (210 mg) is stirred with 0.5 mL m-cresol and 5 mL
15 hydrogen fluoride (HF) at 0°C for 1 hour. After evaporation of the HF under
vacuum, the remnant is washed with dry diethyl ether and ethyl acetate.
The cleaved and deprotected peptide is dissolved in 50% acetic acid and
separated from the resin by filtration. After dilution with water and
lyophilization, 54 mg crude product is obtained.

20

60 mg of the GH-RH antagonist peptide is dissolved in AcOH/H₂O)
and purified by RP-HPLC using the same procedure and equipments
described in Example V. The product is judged to be substantially (>95%)
pure by analytical HPLC. R_t = 13.52 min and k' = 0.819 (isocratic elution
25 with 52% B). Confirmation of the structure is provided by amino acid
analysis.

Peptides 19, 20, 21 and 38 are synthesized in the same manner as
Peptide 18 except that they are acylated with the appropriate anhydride of
30 isobutyric acid, bromopropionic acid, iodoacetic acid or formic acid
respectively in place of Nac, to yield:

[Ibu⁰,D-Arg²,Phe(pCl)⁶,Abu¹⁵,Nle²⁷]hGH-RH(1-28)Agm Peptide 19
 [BrProp⁰,D-Arg²,Phe(pCl)⁶,Abu¹⁵,Nle²⁷]hGH-RH(1-28)Agm Peptide 20
 [IAc⁰,D-Arg²,Phe(pCl)⁶,Abu¹⁵,Nle²⁷]hGH-RH(1-28)Agm Peptide 21
 [For⁰,D-Arg²,Phe(pCl)⁶,Abu¹⁵,Nle²⁷]hGH-RH(1-28)Agm Peptide 38.

5

Peptides 23, 24, 25 and 26 are synthesized in the same manner as Peptide 18 except that Boc-Ser(Bzl)-OH²⁸ is replaced with Boc-Asp(OCHx)-OH²⁸ and they are acylated with the symmetrical anhydride of 1-naphthylacetic acid, 2-naphthylacetic acid, 1-naphthoic acid, and anthraquinone-2-
 10 carboxylic acid, respectively, to yield:

[Nac⁰,D-Arg²,Phe(pCl)⁶,Abu¹⁵,Nle²⁷,Asp²⁸]hGH-RH(1-28)Agm Peptide 23
 [2-Nac⁰,D-Arg²,Phe(pCl)⁶,Abu¹⁵,Nle²⁷,Asp²⁸]hGH-RH(1-28)Agm Peptide 24
 [1-Npt⁰,D-Arg²,Phe(pCl)⁶,Abu¹⁵,Nle²⁷,Asp²⁸]hGH-RH(1-28)Agm Peptide 25
 [Aqc⁰,D-Arg²,Phe(pCl)⁶,Abu¹⁵,Nle²⁷,Asp²⁸]hGH-RH(1-28)Agm Peptide 26.

15

Peptide 22 is synthesized in the same manner as Peptide 23 except that Boc-Tyr(2,6-diCl-Z)-OH¹ is replaced with Boc-His(Bom)-OH¹, to yield:
 [Nac⁰-His¹,D-Arg²,Phe(pCl)⁶,Abu¹⁵,Nle²⁷,Asp²⁸]hGH-RH(1-28)Agm. Peptide 22

20 Peptide 27 is synthesized in the same manner as Peptide 18 except that Boc-Abu-OH¹⁵ is replaced with Boc-Ala-OH¹⁵, to yield:
 [Nac⁰,D-Arg²,Phe(pCl)⁶,Ala¹⁵,Nle²⁷]hGH-RH(1-28)Agm Peptide 27.

Peptide 28 is synthesized in the same manner as Peptide 23 except
 25 that Boc-Asp(OCHx)-)H³ is replaced with Boc-Gly-OH³ to yield:
 [Nac⁰,D-Arg²,Gly³,Phe(pCl)⁶,Abu¹⁵,Nle²⁷,Asp²⁸]hGH-RH(1-28)Agm Peptide 28.

Peptides 29, 30, 31 and 33 are synthesized in the same manner as Peptide 23 except that Boc-Phe-OH⁶ is replaced with Boc-Pro-OH⁶, Boc-Pro-
 30 OH⁶, Boc-hPhe-OH⁶, and Boc-Ala-OH respectively and acylation is performed using the symmetrical anhydride of iodoacetic acid, isobutyric acid, iodoacetic acid and 1-naphthylacetic acid respectively, to yield:

| | |
|--|-------------|
| [IAc ⁰ ,D-Arg ² ,Pro ⁶ ,Abu ¹⁵ ,Nle ²⁷ ,Asp ²⁸]hGH-RH(1-28)Agm | Peptide 29 |
| [Ibu ⁰ ,D-Arg ² ,Pro ⁶ ,Abu ¹⁵ ,Nle ²⁷ ,Asp ²⁸]hGH-RH(1-28)Agm | Peptide 30 |
| [IAc ⁰ ,D-Arg ² ,hPhe ⁶ ,Abu ¹⁵ ,Nle ²⁷ ,Asp ²⁸]hGH-RH(1-28)Agm | Peptide 31 |
| [Nac ⁰ ,D-Arg ² ,Ala ⁶ ,Abu ¹⁵ ,Nle ²⁷ ,Asp ²⁸]hGH-RH(1-28)Agm | Peptide 33. |

5

Peptide 32 is synthesized in the same manner as Peptide 18 except that Boc-Phe(pCl)-OH⁶ is replaced with Boc-Nal-OH⁶ to yield:

[Nac⁰,D-Arg²,Nal⁶,Abu¹⁵,Nle²⁷]hGH-RH(1-28)Agm Peptide 32.

- 10 Peptide 34 is synthesized in the same manner as Peptide 18 except that Boc-D-Arg(Tos)-OH² is replaced with Boc-D-Cit-OH², to yield:
[Nac⁰,D-Cit²,Phe(pCl)⁶, Abu¹⁵,Nle²⁷]hGH-RH(1-28)Agm.

- Peptide 35 is synthesized in the same manner as Peptide 34 except
15 that acylation with the anhydride of 1-naphthylacetic acid is omitted to yield:

[D-Cit²,Phe(pCl)⁶, Abu¹⁵,Nle²⁷]hGH-RH(1-28)Agm.

- Peptide 36 is synthesized in the same manner as Peptide 34 except
20 that Boc-Phe(pCl)-OH⁶ is replaced with Boc-Nal-OH respectively, to yield:
[Nac⁰,D-Cit²,Nal⁶,Abu¹⁵,Nle²⁷]hGH-RH(1-28)Agm.

- Peptide 37 is synthesized in the same manner as Peptide 18 except that after removal of the Boc protecting group from the alpha amino group
25 of Tyr¹, the peptide is *not* acylated.

EXAMPLE VIII

Biological Activity

- The peptides of the present invention were tested in an in vitro and
30 in vivo assay for their ability to inhibit the hGH-RH(1-29)NH₂ induced GH release.

Superfused Rat Pituitary System. The analogues were tested in vitro in a test described earlier (S. Vigh and A.V. Schally, Peptides 5:241-347, 1984) with modification (Z. Rekasi and A.V. Schally, P.N.A.S. 90:2146-2149, 1993).

5

Briefly, the cells are preincubated with peptides for 9 minutes (3mL) at various concentrations. Immediately after the incubation, 1 nM hGH-RH(1-29)NH₂ is administered for 3 minutes (1mL) [0 minute response]. To check the duration of the antagonistic effect of the analogue, 1 nM hGH-
10 RH(1-29)NH₂ is applied 30, 60, 90, and 120 minutes later for 3 minutes [30, 60, 90, 120 min responses]. Net integral values of the GH responses are evaluated. GH responses are compared to and expressed as percent of the original GH response induced by 1 nM GH-RH(1-29)NH₂. The effect of the new antagonists are compared to that of [Ac-Tyr¹,D-Arg²]hGH-RH(1-
15 29)NH₂, the "Standard antagonist".

Growth Hormone Radio-immunoassay. Rat GH levels in aliquots of undiluted and diluted superfusion samples were measured by double-antibody radioimmunoassay using materials supplied by the National
20 Hormone and Pituitary Program, Baltimore, Maryland. The results of RIA were analyzed with a computer program developed in our institute (V. Csernus and A.V. Schally, Harwood Academic (Greenstein, B.C. ed., London, pp. 71-109, 1991), hereby incorporated by reference. Inter-assay variation was less than 15% and intra-assay variation was less than 10%.

25

GH-RH Binding Assay. A sensitive radioreceptor binding assay was developed to determine the binding characteristics of the antagonists of GH-RH (G. Halmos, A.V. Schally et al., Receptor 3, 87-97, 1993), hereby incorporated by reference. The assay is based on binding of labelled
30 [His¹,Nle²⁷]hGH-RH(1-32)NH₂ to rat anterior pituitary membrane homogenates. Iodinated derivatives of [His¹,Nle²⁷]hGH-RH(1-32)NH₂ are

prepared by the chloramine-T method (F.C. Greenwood et al., Biochemistry 89:114-123, 1963), hereby incorporated by reference. Pituitaries from male Sprague-Dawley rats (250-300 g) are used to prepare crude membranes. For saturation binding analyses, membrane homogenates are
5 incubated with at least 6 concentrations of [His¹, ¹²⁵I-Tyr¹⁰, Nie²⁷]hGH-RH(1-32) NH₂, ranging from 0.005 to 0.35 nM in the presence or absence of excess unlabelled peptide (1 μ M). The pellet is counted for radioactivity in a γ -counter. The affinities of the antagonist peptides tested to rat pituitary GH-RH receptors are determined in competitive binding experiments. The
10 final binding affinities are estimated by K_i (dissociation constant of the inhibitor-receptor complex) and are determined by the Ligand PC computer program of Munson and Rodbard as modified by McPherson. Relative affinities compared to [Ac-Tyr¹, D-Arg²]hGH-RH(1-29)NH₂, the Standard antagonist, are calculated as the ratio of K_i of the tested GH-RH antagonist
15 to the K_i of the Standard antagonist.

In Vivo Tests. Adult male Sprague-Dawley rats are anesthetized with pentobarbital (6mg/100g b.w., i.p.). Blood samples are taken from the jugular vein 30 min after the injection of pentobarbital. One group of 7
20 animals receives hGH-RH(1-29)NH₂ as control. Other groups of rats are injected with [Ac-Tyr¹, D-Arg²]hGH-RH(1-29) NH₂ as Standard antagonist, or with one of the antagonist peptide 30 seconds prior to hGH-RH (1-29)NH₂, which is administered at dose of 2-3 μ g/kg b.w. Blood samples are taken from the jugular vein 5 and 15 min after the injection of antagonists.
25 GH levels are measured by RIA. Potencies of the antagonists are calculated by the factorial analysis of Bliss and Marks with 95% confidence limits and are based on the doses of 100 and 400 μ g/kg b.w. of the Standard antagonist and 20 and 80 μ g/kg b.w. of the antagonists tested. Statistical significance was assessed by Duncan's new multiple range test.

Results in vitro. The results of the in vitro antagonistic activities tested in superfused rat pituitary system and binding assay are summarized in Table II and Table III, respectively. As can be seen from these data, acylation of the analogues with Nac or Ibu which contain D-Arg² or D-Cit² substitution combined with Phe(pCl)⁶ or Nal⁶, Abu¹⁵, Nle²⁷, and Agm²⁹ cause an immense increase in receptor binding as well as in inhibition of GH release in vitro. Antagonist peptides [Nac⁰,D-Arg²,pCl-Phe⁶,Abu¹⁵, Nle²⁷] hGH-RH(1-29)NH₂ (Peptide 1), [Nac⁰-His¹,D-Arg²,Phe(pCl)⁶,Abu¹⁵, Nle²⁷] hGH-RH(1-29)NH₂ (Peptide 5), Ibu⁰,D-Arg²,Phe (pCl)⁶,Abu¹⁵,Nle²⁷] hGH-RH(1-28)Agm (Peptide 19) and [Nac⁰, D-Arg², pCl-Phe⁶,Abu¹⁵, Nle²⁷] hGH-RH(1-28)Agm (Peptide 18) are the most effective antagonists in vitro. Peptides 1 and 18 are also extremely long acting in vitro: the inhibition of GH release is 90% (30 nM dose) of the control value 4.5 hours after the incubation in case of Peptide 1; and the inhibition of GH release by Peptide 18 is about 96% (30 nM dose) and 48% (3 nM dose) of the control value even 4.5 and 6 hours after the incubation, respectively. The receptor binding affinities of analogues Peptides 1, 5, and 19 are 82.56, 67.08, and 26.18 times greater respectively than that of the standard GH-RH antagonist.

20

Results in vivo. Table IV shows the serum GH levels in rats pretreated with GH-RH antagonists. Peptides 1 and 19 produce a significant greater and longer-lasting inhibition of the GH response to hGH-RH(1-29)NH₂ than the standard antagonist. In vivo experiments, Peptide 19 inhibits hGH-RH(1-29)NH₂-induced GH-release to greater extent and for a longer period of time than Peptide 1.

25

TABLE II

Inhibition of GH Release in Superfused Rat Pituitary System

| Peptide | Dose (nM) | Inhibition of GH release (%) | | | |
|---------|----------------------|------------------------------|--------|--------|---------|
| | | 0 min | 30 min | 60 min | 120 min |
| 5 | Standard antagonist: | 100 | 62.1 | 2.5 | 19 |
| 10 | 1 | 100 | 23.3 | 93.9 | 89.3 |
| | | 30 | 96.1 | 95 | 92.1 |
| | | 10 | 90.3 | 90 | 87.1 |
| 15 | | 3 | 18.1 | 31.5 | 17.1 |
| | 2 | 30 | 23.1 | 6 | |
| | 3 | 30 | 80.7 | 16.4 | 0 |
| 20 | 4 | 30 | 0 | 0 | 0 |
| | 5 | 30 | 92.6 | 86.4 | 81.4 |
| 25 | 6 | 30 | 17.9 | 0 | 64.5 |
| | 7 | 100 | 73.9 | 25 | 45 |
| | | 10 | 14.2 | 20.8 | 51.6 |
| 30 | 8 | 30 | 59.1 | 0 | 7.3 |
| | 9 | 100 | 90.7 | 79.5 | 76 |
| 35 | | 30 | 88.4 | 46.5 | 43.9 |
| | 10 | 300 | 2.5 | 21.5 | 32.1 |
| | | 100 | 29.4 | 49.7 | |
| 40 | 11 | 300 | 15.8 | 22.3 | |
| | 12 | 100 | 87.9 | 51.8 | 42.4 |
| 45 | | 30 | 81 | 35.6 | 0 |
| | | 10 | 65.5 | 33.6 | 8.5 |
| | 13 | 100 | 87.9 | 63.6 | 51.3 |
| 50 | | 30 | 64.1 | 17.5 | 21 |
| | | 10 | 25.3 | 1.3 | 4.9 |

37

| | Peptide | Dose (nM) | Inhibition of GH release (%) | | | |
|----|---------|--------------|------------------------------|--------|--------|---------|
| | | | 0 min | 30 min | 60 min | 120 min |
| 5 | 14 | 100 | 38.9 | | | |
| | 15 | 100 | 83.6 | 60.2 | 60.3 | |
| | | 30 | 57.2 | 8.4 | 1.9 | |
| 10 | | 10 | 4.5 | 12.8 | 0 | |
| | 16 | 100 | 7.8 | 18.7 | 14.7 | |
| 15 | 17 | 30 | 43.3 | 39.3 | 35.9 | |
| | 18 | 30 | 83.6 | 93.9 | 89.3 | 98.9 |
| | | 10 | 96.6 | 97.2 | 97.1 | 90.0 |
| 20 | | 3 | 77.6 | 83.4 | 75.3 | 58.8 |
| | | 1 | 56.3 | 56.7 | 41.3 | 45.8 |
| 25 | | 0.3 | 11.0 | 45.0 | 15.6 | 13.5 |
| | 19 | 100 | 95 | 74.7 | 36.7 | |
| | | 30 | 82.7 | 40.7 | 9.6 | |
| 30 | | 10 | 70 | 18.2 | 13.9 | |
| | | 3 | 62 | 16.4 | | |
| 35 | 20 | 100 | 86.4 | 75 | 62.8 | |
| | | 30 | 58 | 19.3 | 35.3 | |
| | | 10 | 56.2 | 35.2 | 51.8 | |
| 40 | 21 | 300 | 89.3 | 32.9 | | |
| | 22 | 30 | 98.9 | 8.2 | 53.2 | |
| 45 | | 3 | 45.3 | 12.4 | 25.1 | |
| | 23 | 30 | 89.3 | 85.1 | 71.6 | 63.8 |
| | | 3 | 51.5 | 56.6 | 32.5 | |
| 50 | 24 | 30 | 83.6 | 64.4 | 6.7 | 60 |
| | | 3 | 0 | 33.3 | 0 | |
| 55 | 25 | 30 | 84.5 | 32 | 42.7 | 32.8 |

| Peptide | Dose (nM) | Inhibition of GH release (%) | | | |
|---------|--------------|------------------------------|--------|--------|---------|
| | | 0 min | 30 min | 60 min | 120 min |
| 5 26 | 30 | 64.9 | 48.9 | 42.3 | |
| | 3 | 24 | 31.2 | 21.6 | |
| 28 | 30 | 41.8 | 38.7 | 44 | 41.3 |
| 10 | 3 | 0 | 22.1 | 5.1 | |
| 29 | 100 | 0 | 0 | | |
| 30 | 300 | 36.2 | | | |
| 15 32 | 30 | 87.3 | 88.3 | 75.9 | 71.8 |
| | 3 | 35.9 | 37.1 | 43.4 | |
| 20 33 | 30 | 28.5 | 20.1 | 3.8 | |
| 34 | 30 | 91.2 | 87.4 | 84.8 | |
| 25 | 3 | 70.4 | 50.5 | 40.6 | |
| | 30 | 59.3 | 39.5 | 22.3 | |
| 37 | 30 | 97.5 | 67.3 | 58.4 | 62.1 |
| 30 | 3 | 78.5 | 38.8 | | |
| 38 | 30 | 94.5 | 0 | | |
| 35 | 3 | 49.2 | 0 | | |

TABLE III
K_i values and relative affinities (R.A) of hGH-RH antagonists

| Peptide | K _i (nM) | R.A. |
|----------|---------------------|-------|
| Standard | 3.22 ± 0.12 | 1 |
| 5 1 | 0.04 ± 0.01 | 82.56 |
| 5 | 0.05 ± 0.01 | 67.08 |
| 7 | 1.35 ± 0.02 | 2.39 |
| 8 | 0.91 ± 0.01 | 3.54 |
| 9 | 0.87 ± 0.1 | 3.72 |
| 10 12 | 0.30 ± 0.15 | 10.73 |
| 13 | 0.78 ± 0.06 | 4.13 |
| 15 | 0.73 ± 0.05 | 4.44 |
| 19 | 0.12 ± 0.04 | 26.18 |
| 20 | 0.99 ± 0.12 | 3.27 |
| 15 | | |

TABLE IV

Serum Growth Hormone Levels in Rats Pretreated with Different GH-RH Antagonists
5 Minutes Prior to Stimulation with GH-RH(1-29)NH₂

| 5 | Treatment (intravenously) | Dose (μg/kg) | GH Levels (ng/mL) | POTENCY (measured against the Standard Antagonist) |
|----|------------------------------|-----------------|----------------------|--|
| 10 | Saline | | 89.0 ± 17.7 | |
| | GH-RH(1-29)NH ₂ | 3.0 | 956.7 ± 113.6 | |
| 15 | Standard antagonist | 100.0 | 738.3 ± 34.7 | |
| | | 400.0 | 439.7 ± 47.3* | |
| 20 | Peptide 19 | 20.0 | 451.8 ± 42.2* | |
| | | 80.0 | 155.0 ± 38.2* | |
| 25 | | | | 18.90 95% Limits - 11.0-32.47 |
| 30 | Peptide 1 | 20.0 | 641.2 ± 81.4 | |
| | | 80.0 | 470.0 ± 46.1* | |
| | | | | 6.09 95% Limits -3.11-11.96 |

* p,0.01 vs GH-RH(1-29)NH₂; Potencies of the antagonists were calculated by the factorial analysis of Bliss and Marks.

EXAMPLE IX

5 The experiment of Example VIII is repeated to evaluate the efficacy and duration of effect of GH-RH antagonist Peptide 18 in suppressing GH-RH(1-29)-stimulated serum growth hormone release in rats. Male Sprague-Dawley rats weighing 300-350 g were anesthetized with sodium pentobarbital (50mg/kg b.w.) and half of the initial pentobarbital dosage
10 was given at 45 min intervals to maintain anesthesia. Twenty minutes after injection of pentobarbital, GH-RH antagonist Peptide 18 was administered intravenously in a dose of 80 µg/kg b.w. to the rats (0 time). Nine rats were used in each group. In order to stimulate GH release, bolus iv injections of GH-RH(1-29)NH₂ at a dose of 3µg/kg b.w. were given at 0 time
15 and at 30 min after administration of the GH-RH antagonists. Blood samples were taken from the jugular vein 5 min after GH-RH(1-29)NH₂ injections. Serum GH levels were measured by radio-immunoassay. Statistical significance was assessed by Duncan's new multiple range test. The results of this experiment are shown in Table V.

20

TABLE V

Serum Growth Hormone Levels in Rats

Pretreated with GH-RH Antagonist Peptide 18

5 minutes prior to stimulation with GH-RH(1-29)NH₂ at a dose of 3µg/kg

| 25 | Pretreatment (intravenously) | Dose (µg/kg) | GH Levels (ng/ml) |
|----|---------------------------------|-----------------|----------------------|
| | Saline | | 10.6 ± 0.02 |
| 30 | GH-RH(1-29)NH ₂ | 3.0 | 1650.6 ± 182.7 |
| | Peptide 18 | 80.0 | 1231.3 ± 81.3* |

35 * p < 0.05 vs GH-RH(1-29)NH₂

GH-RH antagonist Peptide 18 injected at a dose of 80 μ g/kg inhibited GH-RH(1-29)NH₂-induced GH secretion by about 24% 5 minutes after its administration.

5

EXAMPLE X

Investigation of the Effect of Peptide 19 on the Growth of Human Osteosarcoma Cell Lines SK-ES-1 and MNNG/HOS Transplanted Athymic Nude Mice or Cultured In Vitro.

Methods: Male athymic nude mice bearing subcutaneously implanted Peptide 19 and MNNG/HOS tumors were treated for 4 and 3 weeks, respectively, with Peptide 19 administered from osmotic minipumps at a dose of 40 μ g/animal/day. Tumor volume and weight, mitotic index, apoptosis and Bromodeoxyuridine (BUDR) labeling index, an indicator of tumor cell proliferation were determined. The effect of Peptide 19 on IGF-I levels in serum, tumor and liver tissue were measured. Concentration of receptors of IGF-I was determined in tumor membrane fractions of both osteosarcomas. In addition, direct effects of Peptide 19 on DNA synthesis and proliferation of SK-ES-1 and MNNG/HOS cells, as well as on the secretion of IGF-I by these cell lines were evaluated in cell cultures.

Results: Growth of both osteosarcomas in nude mice was significantly inhibited by Peptide 19 (Figs. 1 and 2). Growth inhibition of SK-ES-1 and MNNG/HOS tumors were reflected by a reduction in tumor volume of 64% and 49%, and a reduction in tumor weight of 62% and 47% respectively, after treatment with Peptide 19. Therapy with Peptide 19 also decreased tumors by 76% as measured by specific RIA. Receptor analyses demonstrated high affinity binding sites for IGF-I on membranes of both tumors. The concentration of IGF-I in liver tissue of nude mice not bearing tumors injected daily for 5 days with Peptide 19 was decreased by about 40% as compared to that in untreated animals. In cell cultures, the proliferation of SK-ES-1 and MNNG/HOS cells, as well as the [³H]thymidine incorporation into the DNA of both cell lines were strongly inhibited by

antagonist Peptide 19. The GH-RH antagonist also markedly reduced the autocrine secretion of IGF-I by both cell lines in vitro.

EXAMPLE XI

5 Study of the Effects of Peptide 19 on MXT Estrogen Independent Mouse Mammary Tumors

Female BDF mice were transplanted with 1mm 3 pieces of an MXT (3.2) estrogen independent breast cancer. Treatment with Peptide 19 started one day after transplantation as follows:

| | | |
|----|---------------------------------|--------|
| 10 | 0.8 μ g/day s.c. once daily | 5 mice |
| | 3.2 μ g/day s.c. once daily | 5 mice |

Tumor volume was measured on day 10, 14, and 18. The results are shown on the graph (Fig. 3). Antagonist Peptide 19 at either dose significantly inhibited the growth of breast cancer in mice.

15

EXAMPLE XII

Long Acting intramuscular injectable formulation (Sesame Oil Gel)

| | | |
|----|---|-----------|
| | [Nac ⁰ ,D-Arg ² ,Phe(pCl) ⁶ ,Abu ¹⁵ ,Nle ²⁷]hGH-RH(1-29)NH ₂ | |
| | (Peptide 1) | 10.0 mg |
| 20 | Aluminum monostearate, USP | 20.0 mg |
| | Sesame oil g.s. | ad 1.0 ml |

The aluminum monostearate is combined with the sesame oil and heated to 125°C with stirring until a clear yellow solution forms. This mixture is then autoclaved for sterility and allowed to cool. The hGH-RH antagonist Peptide 1 is then added aseptically with trituration. Particularly preferred antagonists are salts of low solubility, e.g., pamoate salts and the like. These exhibit long duration of activity.

EXAMPLE XIII

Aqueous Solution for Intramuscular Injection

| | | |
|---|---|-----------|
| | [Nac ⁰ ,His ¹ -D-Arg ² ,Phe(pCl) ⁶ ,Abu ¹⁵ ,Nle ²⁷]hGH-RH(1-29)NH ₂ | |
| | (Peptide 5) | 500 mg |
| 5 | Gelatin, nonantigenic | 5 mg |
| | Water for injection g.s. | ad 100 ml |

The gelatin and GHRH antagonist Peptide 19 are dissolved in water for injection, then the solution is sterile filtered.

10

EXAMPLE XIV

Long Acting IM Injectable-Biodegradable Polymer Microcapsules

Microcapsules are made from the following:

| | | |
|----|---|-----|
| | 25/75 glycolide/lactide copolymer | |
| 15 | (0.5 intrinsic viscosity) | 99% |
| | [Ibu ⁰ ,D-Arg ² ,Phe(pCl) ⁶ ,Abu ¹⁵ ,Nle ²⁷]hGH-RH(1-28)Agm ²⁹ | |
| | (Peptide 19) | 1% |

20 25 mg of the above microcapsules are suspended in 1.0 ml of the following vehicle:

| | | |
|----|----------------------|--------|
| | Dextrose | 5.0% |
| | CMC, sodium | 0.5% |
| 25 | Benzyl alcohol | 0.9% |
| | Tween 80 | 0.1% |
| | Water, purified q.s. | 100.0% |

30 The test results described herein are generally considered by those skilled in the art to be predictive of results in humans.

SEQUENCE LISTING

(1) GENERAL INFORMATION:

- 5 (i) APPLICANT: The Administrators of the Tulane
Educational Fund
- (ii) TITLE OF INVENTION: ANALOGUES OF hGH-RH(1-29)NH2
10 HAVING
ANTAGONISTIC ACTIVITY
- (iii) NUMBER OF SEQUENCES: 2
- (iv) CORRESPONDENCE ADDRESS:
15 (A) ADDRESSEE: OMRI M. BEHR, ESQ
(B) STREET: 325 PIERSON AVENUE
(C) CITY: EDISON
(D) STATE: NEW JERSEY
(E) COUNTRY: USA
20 (F) ZIP: 08837
- (v) COMPUTER READABLE FORM:
(A) MEDIUM TYPE: Floppy disk
(B) COMPUTER: IBM PC compatible
25 (C) OPERATING SYSTEM: PC-DOS/MS-DOS
(D) SOFTWARE: PatentIn Release #1.0, Version
#1.25
- (vi) CURRENT APPLICATION DATA:
30 (A) APPLICATION NUMBER:
(B) FILING DATE:
(C) CLASSIFICATION:
- (viii) ATTORNEY/AGENT INFORMATION:
35 (A) NAME: BEHR, OMRI M.
(B) REGISTRATION NUMBER: 22,940
(C) REFERENCE/DOCKET NUMBER: SHAL 3.0-020
- (ix) TELECOMMUNICATION INFORMATION:
40 (A) TELEPHONE: (908) 494-5240
(B) TELEFAX: (908) 494-0428]
(C) TELEX: 511642 BEPATEDIN
- 45 (2) INFORMATION FOR SEQ ID NO:1:
- (i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 29 amino acids
(B) TYPE: amino acid
50 (C) STRANDEDNESS: single
(D) TOPOLOGY: linear
- (ii) MOLECULE TYPE: peptide
- 55 (v) FRAGMENT TYPE: N-terminal

45

(ix) FEATURE:

(A) NAME/KEY: misc_feature

(B) LOCATION: 29

(D) OTHER INFORMATION: /note= "Res 29 = Arg-NH₂"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

Tyr Ala Asp Ala Ile Phe Thr Asn Ser Tyr Arg Lys Val Gln Leu
1 5 10 15

Gly Gln Leu Ser Ala Arg Lys Leu Leu Gln Asp Ile Met Ser Xaa
20 25

(2) INFORMATION FOR SEQ ID NO:2:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 29 amino acids

(B) TYPE: amino acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(v) FRAGMENT TYPE: N-terminal

(ix) FEATURE:

(A) NAME/KEY: misc_feature

(B) LOCATION: 1

(D) OTHER INFORMATION: /note= "Res 1 = Tyr or His"

(ix) FEATURE:

(A) NAME/KEY: misc_feature

(B) LOCATION: 2

(D) OTHER INFORMATION: /note= "Res 2 = substituted D-Arg residues"

(ix) FEATURE:

(A) NAME/KEY: misc_feature

(B) LOCATION: 27

(D) OTHER INFORMATION: /note= "Res 27 = Nle"

(ix) FEATURE:

(A) NAME/KEY: misc_feature

(B) LOCATION: 29

(D) OTHER INFORMATION: /note= "Res 29 = Arg-NH₂"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

Xaa Xaa Asp Ala Ile Phe Thr Asn Ser Tyr Arg Lys Val Leu Gly
5 1 5 10 15

Gln Leu Ser Ala Arg Lys Leu Leu Gln Asp Ile Xaa Ser Xaa
20 25

CLAIMS

1. A peptide having the formula:
X-R¹-R²-R³-R⁴-R⁵-R⁶-Thr-R⁸-Ser-Tyr-R¹¹-R¹²-Val-Leu-R¹⁵-Gln-Leu-Ser-R¹⁸-R²⁰-
5 R²¹-Leu-Leu-Gln-Asp-Ile-R²⁷-R²⁸-R²⁹ wherein
X is nil, H, Ac, IAc, BrProp, For, Ibu, Nac, 2-Nac, 1- or 2-Npt, 1- or 2-Npr
or Aqc
R¹ is Tyr, His, Glt or Glu,
R² is D-Arg, D-Cit, D-Har, D-Lys or D-Orn,
10 R³ is Asp, Ala or Gly,
R⁴ is Ala or Gly,
R⁵ is Ile, Ala or Gly,
R⁶ is Phe, Ala, Pro, Tpi, Nal or Phe(Y), in which Y is F, Cl, Br, NO₂, CH₃ or
OCH₃,
15 R⁸ is Asn, Ser, Val, Ile, Ala, Abu, Nle, or Aib,
R¹¹ is Arg, D-Arg or Cit,
R¹² is Lys, D-Lys, Cit or Ala,
R¹⁵ is Gly, Ala, Abu or Gln,
R¹⁸ is Ala or Abu,
20 R²⁰ is Arg, D-Arg or Cit,
R²¹ is Lys, D-Lys or Cit,
R²⁷ is Met, Nle or Abu,
R²⁸ is Ser, Asn, Asp or Abu,
R²⁹ is Agm, Arg-NH₂, Arg-OH, Cit-NH₂, Cit-OH, Har-NH₂ or Har-OH,
25 provided that when R¹ is Glt, X is nil and when X is H, R¹⁵ is other than Gly,
and pharmaceutically acceptable acid addition salts thereof.

2. A peptide according to Claim 1 wherein
30 X is H, Ac, IAc, BrProp, For, Ibu, Nac, 2-Nac, 1- or 2-Npt, 1- or 2-Npr, or
Aqc,

- R¹ is Tyr, His, or Glu,
R² is D-Arg or D-Cit,
R³ is Asp or Gly,
R⁶ is Phe, Phe(pCl), Tpi, Pro, hPhe, Nal or Ala,
5 R⁸ is Asn or Aib,
R¹² is Lys or Ala,
R¹⁵ is Abu or Ala,
R¹⁹ is Ala or Abu,
R²⁷ is Nle,
10 R²⁸ is Ser or Asp,
R²⁹ is Arg-NH₂ and pharmaceutically acceptable acid addition salts thereof.

3. A peptide according to Claim 2 wherein
15 X is H, Ibu, For, Nac, 2-Nac, or 1-Npt,
R¹ is Tyr, His or Glu,
R² is D-Arg,
R³ is Asp,
R⁶ is Phe(pCl), Tpi, or Nal,
20 R⁸ is Asn,
R¹² is Lys,
R¹⁵ is Abu,
R¹⁹ is Ala, and
R²⁸ is Ser.

25

4. A peptide according to Claim 3 wherein R⁶ is Phe(pCl).

5. A peptide according to Claim 4 wherein X is Ibu or Nac, and
R¹ is Tyr or His.

30

6. A peptide according to Claim 5 wherein X is Nac and R¹ is Tyr.

7. A peptide according to Claim 5 wherein X is Ibu.
8. A peptide according to Claim 5 wherein R¹ is His.
- 5 9. A peptide according to Claim 2 wherein X is Nac, R² is D-Cit and R²⁹ is Agm.
- 10 10. A peptide according to Claim 3 wherein X is Nac, R⁶ is Nal and R²⁹ is Agm.
11. A peptide according to Claim 3, wherein X is For.
12. A peptide according to Claim 4 selected from the group consisting of peptides of the formula:
- 15 Nac⁰-Tyr-D-Arg²-Asp-Ala-Ile-Phe(pCl)⁶-Thr-Asn-Ser-Tyr-Arg-Lys-Val-Leu-
Abu¹⁵-Gln-Leu-Ser-Ala-Arg-Lys-Leu-Leu-Gln-Asp-Ile-Nle²⁷-Ser-Arg-NH₂,
Nac⁰-His¹-D-Arg²-Asp-Ala-Ile-Phe(pCl)⁶-Thr-Asn-Ser-Tyr-Arg-Lys-Val-Leu-
Abu¹⁵-Gln-Leu-Ser-Ala-Arg-Lys-Leu-Leu-Gln-Asp-Ile-Nle²⁷-Ser-Arg-NH₂,
Ibu⁰-Tyr-D-Arg²-Asp-Ala-Ile-Phe(pCl)⁶-Thr-Asn-Ser-Tyr-Arg-Lys-Val-Leu-
20 Abu¹⁵-Gln-Leu-Ser-Ala-Arg-Lys-Leu-Leu-Gln-Asp-Ile-Nle²⁷-Ser-Agm, and
For⁰-Ibu⁰-Tyr-D-Arg²-Asp-Ala-Ile-Phe(pCl)⁶-Thr-Asn-Ser-Tyr-Arg-Lys-Val-Leu-
Abu¹⁵-Gln-Leu-Ser-Ala-Arg-Lys-Leu-Leu-Gln-Asp-Ile-Nle²⁷-Ser-Agm.
13. A peptide according to Claim 12 having the formula
- 25 Nac⁰-Tyr-D-Arg²-Asp-Ala-Ile-Phe(pCl)⁶-Thr-Asn-Ser-Tyr-Arg-Lys-Val-Leu-
Abu¹⁵-Gln-Leu-Ser-Ala-Arg-Lys-Leu-Leu-Gln-Asp-Ile-Nle²⁷-Ser-Arg-NH₂.
14. A peptide according to Claim 12 having the formula
- 30 Nac⁰-His¹-D-Arg²-Asp-Ala-Ile-Phe(pCl)⁶-Thr-Asn-Ser-Tyr-Arg-Lys-Val-Leu-
Abu¹⁵-Gln-Leu-Ser-Ala-Arg-Lys-Leu-Leu-Gln-Asp-Ile-Nle²⁷-Ser-Arg-NH₂.

15. A peptide according to Claim 12 having the formula
Ibu⁰-Tyr-D-Arg²-Asp-Ala-Ile-Phe(pCl)⁶-Thr-Asn-Ser-Tyr-Arg-Lys-Val-Leu-
Abu¹⁵-Gln-Leu-Ser-Ala-Arg-Lys-Leu-Leu-Gln-Asp-Ile-Nle²⁷-Ser-Agm.

5 16. A peptide according to Claim 12 having the formula
For⁰-Ibu⁰-Tyr-D-Arg²-Asp-Ala-Ile-Phe(pCl)⁶-Thr-Asn-Ser-Tyr-Arg-Lys-Val-Leu-
Abu¹⁵-Gln-Leu-Ser-Ala-Arg-Lys-Leu-Leu-Gln-Asp-Ile-Nle²⁷-Ser-Agm.

10 17. A peptide according to Claim 3 selected from the group
consisting of peptides of the formula
Nac⁰-Tyr-D-Arg²-Asp-Ala-Ile-Phe(pCl)⁶-Thr-Asn-Ser-Tyr-Arg-Lys-Val-Leu-
Abu¹⁵-Gln-Leu-Ser-Ala-Arg-Lys-Leu-Leu-Gln-Asp-Ile-Nle²⁷-Ser-Agm,
Nac⁰-Tyr-D-Arg²-Asp-Ala-Ile-Nal⁶-Thr-Asn-Ser-Tyr-Arg-Lys-Val-Leu-Abu¹⁵-
Gln-Leu-Ser-Ala-Arg-Lys-Leu-Leu-Gln-Asp-Ile-Nle²⁷-Ser-Agm, and
15 Nac⁰-Tyr-D-Cit²-Asp-Ala-Ile-Phe(pCl)⁶-Thr-Asn-Ser-Tyr-Arg-Lys-Val-Leu-
Abu¹⁵-Gln-Leu-Ser-Ala-Arg-Lys-Leu-Leu-Gln-Asp-Ile-Nle²⁷-Ser-Agm.

18. A peptide according to Claim 17 having the formula
Nac⁰-Tyr-D-Arg²-Asp-Ala-Ile-Phe(pCl)⁶-Thr-Asn-Ser-Tyr-Arg-Lys-Val-Leu-
20 Abu¹⁵-Gln-Leu-Ser-Ala-Arg-Lys-Leu-Leu-Gln-Asp-Ile-Nle²⁷-Ser-Agm.

19. A peptide according to Claim 17 having the formula
Nac⁰-Tyr-D-Arg²-Asp-Ala-Ile-Nal⁶-Thr-Asn-Ser-Tyr-Arg-Lys-Val-Leu-Abu¹⁵-
Gln-Leu-Ser-Ala-Arg-Lys-Leu-Leu-Gln-Asp-Ile-Nle²⁷-Ser-Agm.

25

20. A peptide according to Claim 17 having the formula
Nac⁰-Tyr-D-Cit²-Asp-Ala-Ile-Phe(pCl)⁶-Thr-Asn-Ser-Tyr-Arg-Lys-Val-Leu-
Abu¹⁵-Gln-Leu-Ser-Ala-Arg-Lys-Leu-Leu-Gln-Asp-Ile-Nle²⁷-Ser-Agm.

30

21. The use of a peptide according to Claim 1 for treating diabetic
retinopathy.

22. The use of a peptide according to Claim 1 for treating diabetic nephropathy.

23. The use of a peptide according to Claim 1 for treating
5 acromegaly.

24. The use of a peptide according to Claim 1 for treating the growth of MXT estrogen independent mouse mammary cancer.

10 25. The use of a peptide according to Claim 1 for treating human osteosarcomas.

26. A composition comprising a pharmaceutically effective amount of a peptide according to Claim 1 in a pharmaceutically acceptable carrier.

1 / 3

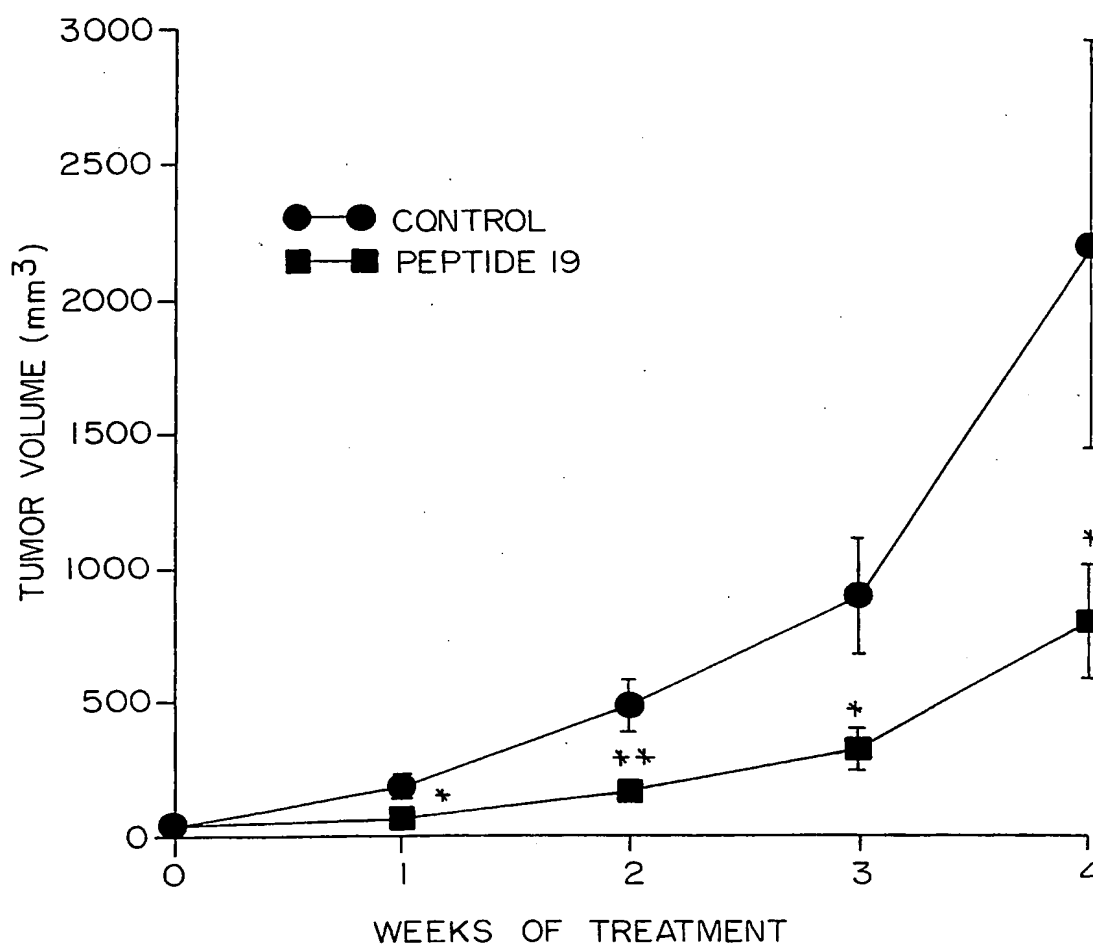


FIG. 1

SUBSTITUTE SHEET (RULE 26)

2 / 3

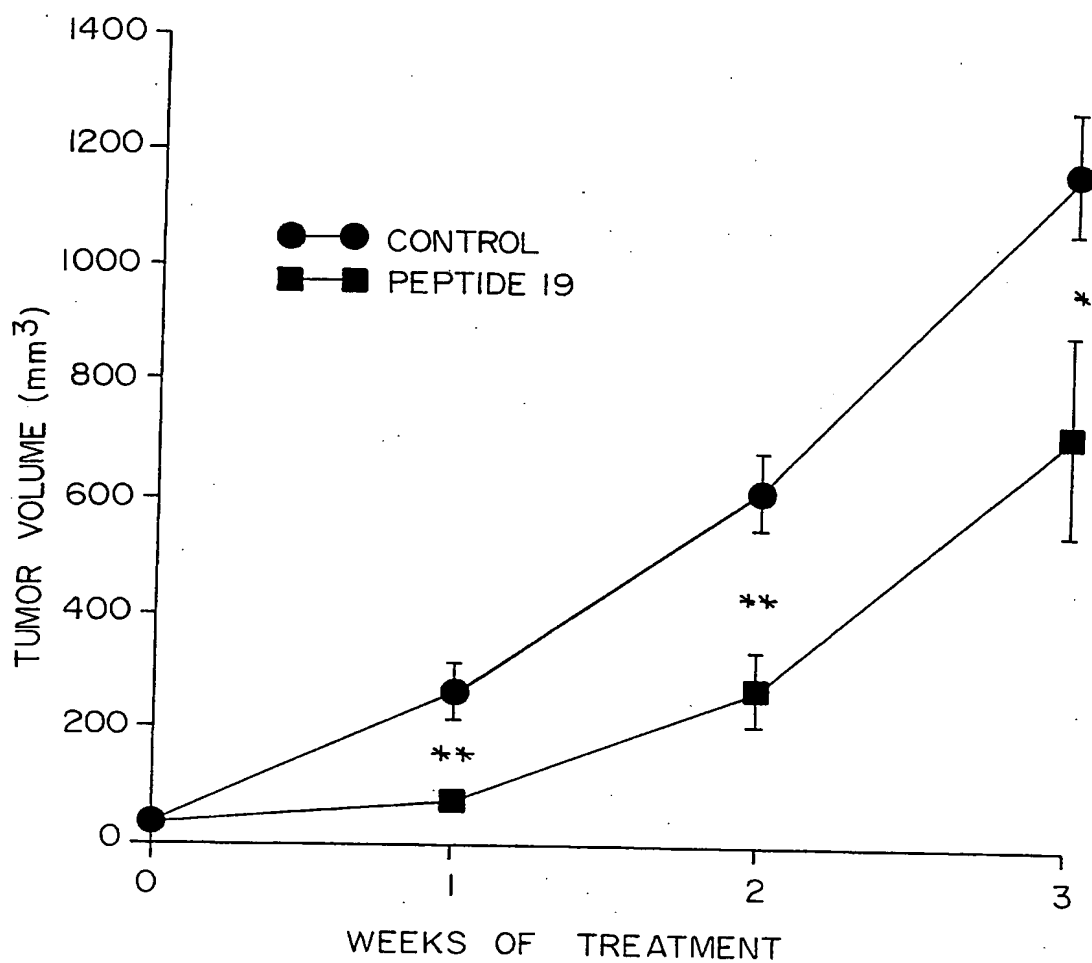


FIG. 2

SUBSTITUTE SHEET (RULE 26)

3 / 3

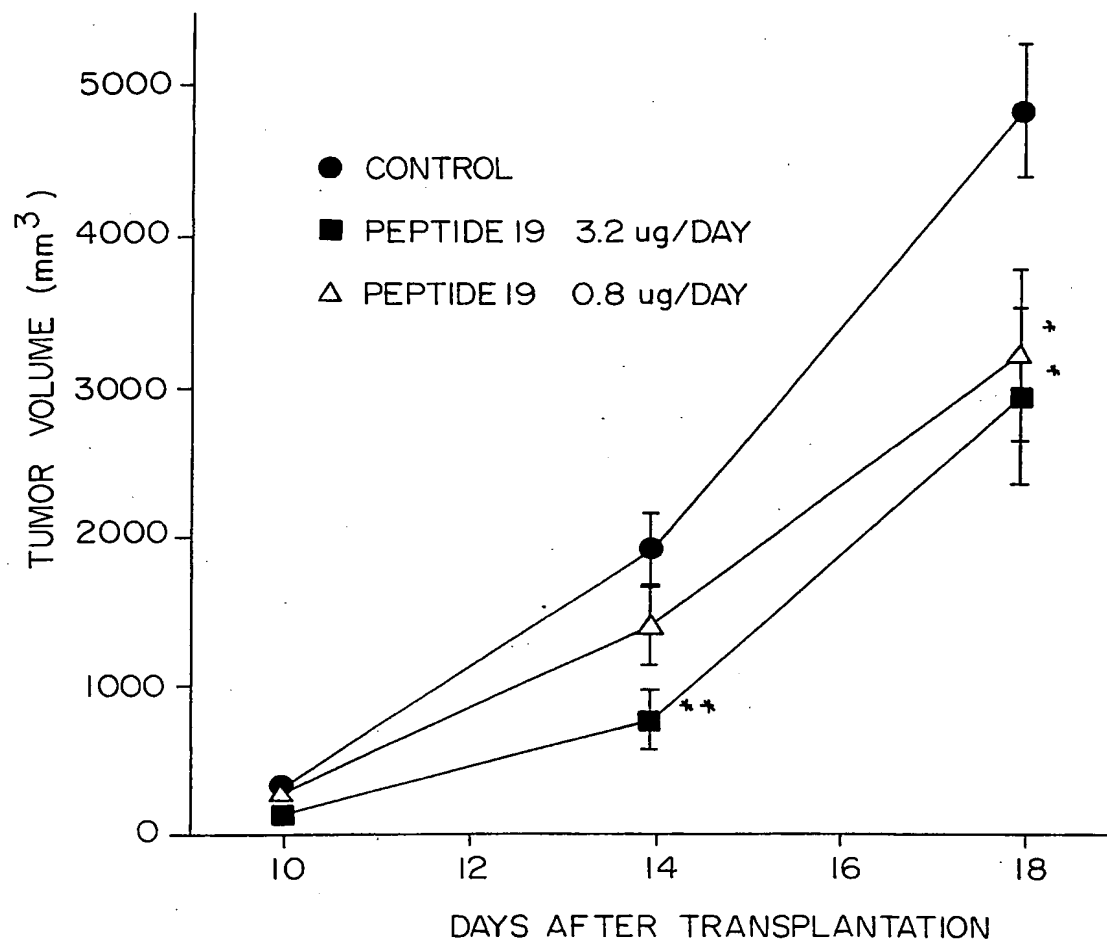


FIG. 3

INTERNATIONAL SEARCH REPORT

International Application No
PCT/US 94/13714

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 C07K14/60 A61K38/25

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC 6 C07K A61K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

| Category * | Citation of document, with indication, where appropriate, of the relevant passages | Relevant to claim No. |
|------------|--|-----------------------|
| X | WO,A,91 16923 (THE ADMINISTRATORS OF THE TULANE UNIVERSITY EDUCATIONAL FUND) 14 November 1991 cited in the application see the whole document --- | 1,2, 21-26 |
| X | G.R.MARSHALL 'PEPTIDES,CHEMISTRY AND BIOLOGY; Proc.10th Am.Pept.Symp.,May 23-28,1987,St.Louis' 1988 , ESCOM , LEIDEN N.Ling et al: "Growth hormone-releasing factor analogs with potent antagonist activity" see page 484 - page 486 --- -/-- | 1,2, 21-26 |

☒ Further documents are listed in the continuation of box C.

☒ Patent family members are listed in annex.

* Special categories of cited documents :

- *A* document defining the general state of the art which is not considered to be of particular relevance
- *E* earlier document but published on or after the international filing date
- *L* document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
- *O* document referring to an oral disclosure, use, exhibition or other means
- *P* document published prior to the international filing date but later than the priority date claimed

- *T* later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
- *X* document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
- *Y* document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.
- *&* document member of the same patent family

Date of the actual completion of the international search

11 April 1995

Date of mailing of the international search report

08.05.95

Name and mailing address of the ISA

European Patent Office, P.B. 5818 Patentlaan 2
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Fax: (+ 31-70) 340-3016

Authorized officer

Groenendijk, M

INTERNATIONAL SEARCH REPORT

Inter nal Application No
PCT/US 94/13714

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

| Category * | Citation of document, with indication, where appropriate, of the relevant passages | Relevant to claim No. |
|------------|---|-----------------------|
| X | BIOCHEM.BIOPHYS.RES.COMM, vol. 167,no. 1, 28 February 1990 pages 360-366, K.SATO ET AL 'Synthetic analogs of growth hormone-releasing-factor with antagonistic activity in vitro' see the whole document --- | 1,21-26 |
| A | EP,A,O 413 839 (THE ADMINISTRATORS OF THE TULANE EDUCATIONAL FUND) 27 February 1991 see page 2, line 39 - line 48 ----- | 1-12, 14-26 |

INTERNATIONAL SEARCH REPORT

International application No.

PCT/US 94/ 13714

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☒ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:
Remark: Although claims 21-25 are directed to a method of treatment of the human/animal body the search has been carried out and based on the alleged effects of the compound/composition.
2. ☐ Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:
3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.
2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.
3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:
4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

- ☐ The additional search fees were accompanied by the applicant's protest.
- ☐ No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

Internal Application No
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| Patent document cited in search report | Publication date | Patent family member(s) | Publication date |
|---|---------------------|---|--|
| WO-A-9116923 | 14-11-91 | AU-B- 651976 AU-A- 7882291 EP-A- 0527914 JP-T- 6502618 | 11-08-94 27-11-91 24-02-93 24-03-94 |
| EP-A-413839 | 27-02-91 | NONE | |